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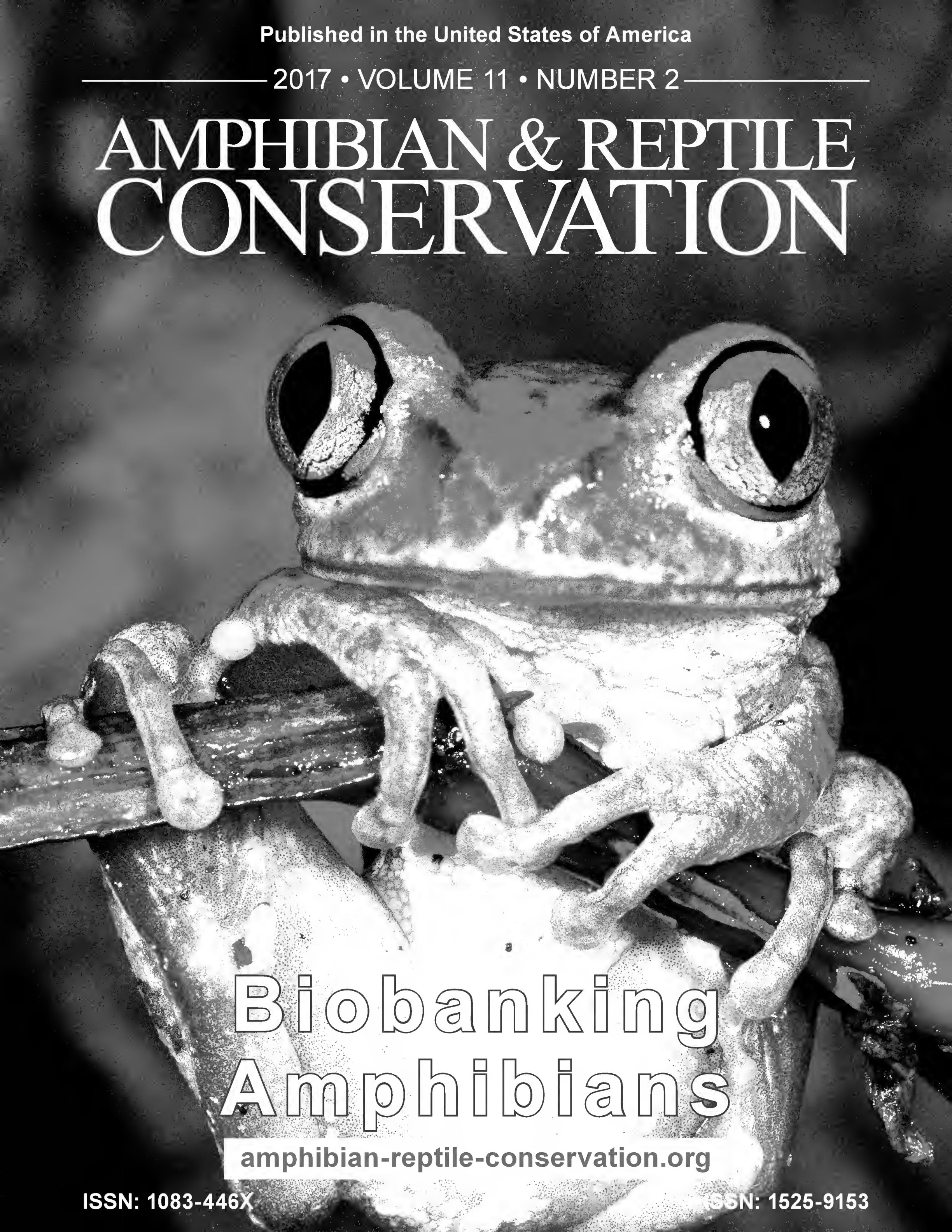

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REPORT

In vitro fertilizations with cryopreserved sperm of *Rhinella marina* (Anura: Bufonidae) in Ecuador

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***Abstract.*—Considering worldwide amphibian population decline, sperm cryopreservation should be a priority for conservation of species in areas of high biodiversity, such as the Neotropics. In this study, we present the results of two cryopreservation experiments involving *Rhinella marina* sperm. Freezing was performed in a -80 °C freezer and dimethyl sulfoxide (DMSO) was used as cryoprotective agent. In the first experiment, the effects of 5%, 10%, and 15% DMSO were evaluated in sperm lysis and fertilization capacity. Samples were incubated for 10 minutes at 4 °C before freezing. For thawing, two procedures were tested: 21 °C thawing to be used immediately and 4 °C thawing, to be used two hours later in *in vitro* fertilizations. The best treatment was 10% DMSO plus thawing at 4 °C, that achieved 20% successful fertilizations. In the second experiment, two solutions were tested: 10% DMSO with and without HEPES. Freezing and post-thawing *in vitro* fertilizations were performed after a two hour incubation period at 4 °C. A considerable improvement in fertilization percentages was obtained in this experiment, with a 75% for DMSO alone, and a 70% for DMSO + HEPES. These results provide good perspectives for future implementation of sperm cryopreservation in Neotropical institutions for local threatened species.**

Keywords. Dimethyl sulfoxide, fertilization percentages, Neotropics, sperm cryopreservation, *in vitro* fertilization, Assisted Reproductive Technologies, toad

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Introduction

The extinction crisis faced by amphibians can be considered as dramatic as that of the Triassic or Cretaceous periods with 31% of species threatened (Kouba et al. 2013). Captive breeding programs (CBP) have been established to ameliorate current amphibian population declines, especially for those species which are faced with poorly understood threats and are rapidly disappearing (Bishop et al. 2012).The aim of dedicated CBP is to maintain *ex situ* populations of target species with high genetic diversity for research and future reintroduction. Assisted reproductive technologies (ART) can be implemented by CBP's when reproduction in captivity is difficult to achieve (Clulow et al. 2014). ART research for amphibians has specialized in gamete collection through hormonal induction, *in vitro* fertilization (IVF), and sperm cryopreservation in several anuran and some caudate species (Bishop et al. 2012). This last technique is very useful because it allows the maintenance of high genetic diversity with a minimum amount of space and resources (Clulow et al. 2014).

Sperm cryopreservation for amphibians still lags behind that of other vertebrate classes (Clulow et al. 2014), though, there are various publications with Pipidae (Sargent and Mohun 2005), Bufonidae (Browne et al. 1998; Beesley et al. 1998), Ranidae (Beesley et al. 1998; Mansour et al. 2010; Mugnano et al. 1998), Eleutherodactyliade (Michael and Jones 2004), Hylidae and Myobatrachidae (Browne et al. 2002) family members. In these studies, testicular sperm is cooled by liquid nitrogen (LN2) quenched in a cooling chamber or by immersion in ethanol/dry ice slurry, and cooling rates determined by a thermocouple. The most commonly reported cryopro-

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Fig. 1. *Rhinella marina* embryo at 31 Gosner stage from *in vitro* fertilization with cryopreserved sperm.

tective agents (CPA) are dimethyl sulfoxide (DMSO) and glycerol at 5%, 10%, 15%, or 20% v/v diluted in saline or sucrose solutions and high temperatures are employed to achieve a fast thawing. However, the effectiveness of the CPA varies according the species and the cryopreservation protocol.

The standardization of a cryopreservation protocol for a species allows its inclusion into genome resource banks (Clulow et al. 2014). Therefore, there is a need to standardize gamete cryopreservation protocols for neotropical species because they comprise approximately 49% of the world's amphibian species and 60% of all threatened species (Bolaños et al. 2008). Moreover, sperm cryopreservation for conservation purposes in this region has focused mainly on fish (Viveiros and Godinho 2009; Carolsfeld et al. 2003) and mammal (Adams et al. 2009) species. To the authors' knowledge, there are only two research papers describing sperm cryopreservation for anuran neotropical species: one published by Michael and Jones (2004) on *Eleutherodactylus coqui*, and the other by Della Togna (2015) on *Atelopus zeteki*.

Here we present two experiments conducted with *Rhinella marina* sperm. This species is abundant in Ecuador and belongs to the Bufonidae family, which encompasses 53% of the threatened species in the Neotropics (Bolaños et al. 2008). Samples were frozen in a -80 °C freezer in plastic racks and DMSO was used as CPA in both experiments. In the first experiment, DMSO was tested at three different concentrations and with two thawing regiments. The second experiment examined the effects of HEPES buffer incorporation into the isotonic solution. HEPES was used in the isotonic solution of our experiments because it is an effective protector of sperm functionality after short term storage in mammals (Will et al. 2011), and it improved sperm motility after 48 h storage in previous trials (unpublished data). Glycerol, the other common CPA, was not used in these experiments, because, at a 10% concentration, it had lower fertilization percentages (13.43 ± 7.42%) than DMSO 10 % (38.50 ± 6.29%) in a previous experiment under similar experimental procedures (unpublished data).

Materials and Methods

General animal and sperm collection

Rhinella marina male and female adults were collected in Jama, Manabí Province, Ecuador (00°11.160'S 080°17.547'W) during the rainy seasons between late December and late March of 2013 and 2015. Six males and four females were collected in the first field trip, and six males and two females in the second one. In both cases, individuals were transported to Pontificia Universidad Católica del Ecuador (PUCE) in Quito, Pichincha Province, Ecuador, and maintained for two weeks in 56.6 L plastic boxes, provided with two water containers and fed crickets twice a week in accordance with Barnett et al. 2001.

For surgical removal of the testicles, individuals were anaesthetized with a 0.5% w/v solution of MS-222 (Sigma-Aldrich E10521-10G), pH 7, for 15–20 minutes (Wright 2001). A half testicle was used in every freezing treatment, thus whole or half testicle was left in the animal to obtain a control sperm suspension (fresh sperm) when IVF was performed. After testicle removal, animals were sutured with Vycril 3-0, and were placed in individual aquaria for recovery.

The testicles were held on ice in suspension buffer (SB: 104.4 mM NaCl, 2 mM KCl, 6.1 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4; Beesley et al. 1998) with HEPES (Gibco 15630-080) at a final concentration of 2.5 mM. The testes for each treatment were bisected and weighed to the nearest 0.03 g. Each half was placed in a 1.5 ml microfuge tube with the corresponding experimental

solution. In all cases, except for the DMSO treatment in experiment two, DMSO was diluted to experimental concentrations in SB with HEPES 2.5 mM. Maceration of testicles was performed with Novo Surgical 0250-22 scissors. The tubes were centrifuged briefly, and the supernatant was placed in another 1.5 ml tube. The resulting sperm suspension was distributed, in different volumes in each experiment, in 600 µl microfuge tubes, and placed in plastic racks for freezing in a -80 °C freezer. The sperm concentration was determined by duplicate counts with an improved Neubauer chamber.

For control sperm solutions in both experiments, the remaining testicle in each animal was removed after euthanasia by administration of the same 0.5% MS-222 solution, but for one and a half hours, and the heart was removed to ensure death (Wright 2001). Testicles were macerated in 1.5 ml microfuge tubes containing SB with HEPES, after a brief centrifugation, supernatant was placed in other 1.5 ml tube and held at 4 °C until use.

Experiment one (E1, n = 6 males). The half testicle was macerated in two ml of any of the following solutions: SB + HEPES, 5%, 10%, or 15% DMSO. DMSO sperm solutions were divided in 250 µl aliquots to be frozen. Samples were maintained 10 minutes at 4 °C and one hour at -20 °C before being placed in a -80 °C freezer. One week later, sperm samples were left in their respective plastic racks until ice melted at room temperature (RT, 21 °C) or at 4 °C. For IVF, sperm samples thawed at RT were used immediately, while sperm samples thawed at 4 °C were used after two hours at 4 °C. Embryos that reached gastrula stage (Gosner's 11 stage) were recorded and a gastrula rate was calculated per petri dish. Sperm counts were made only for RT treatments.

Experiment two (E2, n = 6 males). Half testicle was macerated in 500 µl of SB + HEPES; 10% DMSO; or 10% DMSO + 2.5 mM HEPES. DMSO suspensions were divided into 100 µl aliquots and placed in a plastic rack to be held at 4 °C for two hours before freezing at -80 °C for three days. Thawing procedure at 4 °C from E1 was employed. Embryos at second cleavage (Gosner's 4 stage) were recorded and maintained until tail bud stage (Gosner's 17 stage), cleavage and tail bud rates were calculated per petri dish.

In vitro fertilization

For both experiments, ovulation in females was induced by injection of fresh pituitary homogenate from one female of the same species. Twelve hours after hormone administration, females were euthanized as previously described for males. Two females were induced to ovulation in E1, eggs from one female were used for RT thawing treatment and eggs from the other one, for 4 °C thawing treatment. Eggs from only one female were used for all treatments in E2. Eggs were removed from the oviduct and placed in a petri dish for fertilization. Experiment one (E1) used 100µl of sperm solution for 208 ± 20 eggs, while experiment two (E2) used 50 µl of sperm for 116 ± 18 eggs per petri dish. Sperm suspension was pipetted directly from the fresh or thawed sample onto the eggs without any previous CPA wash or dilution. Around two minutes later, the eggs were covered with six ml of filtered tap water, and after 10 minutes, 20 ml of water were added. Embryos were reared to tail bud stage (Gosner's 17 stage) in 10 cm Petri dishes filled with filtered tap water that was changed daily.

Statistics

Two factor ANOVA and Wilcoxon test were performed for E1 and E2, respectively, using SPSS 20. Gastrula rate data of E1 were analyzed by CPA and thawing procedure factors. Cleavage rates within each DMSO treatment of E2 were analyzed by a Wilcoxon test because data size was lower than 30 samples. $\alpha = 0.05$ for both analyses.

Results and Discussion

In both experiments, IVF's with cryopreserved sperm resulted in embryo development that reached tail bud stage, although different embryo survival rates were achieved in each experiment. DMSO 10% + HEPES 2.5 mM treatment was present in both experiments and had 20% embryos in E1, and 54% in E2. These slower embryo rates in E1 could be due to the freezing procedure, which may allowed melting and recrystallization when moving samples from 4 °C to -20 °C and from -20 °C to -80 °C freezers. Besides, it is important to take into consideration factors such as the different sperm concentration, the frozen volume and the pre-freezing DMSO incubation period in E2.

DMSO 10% with 4 °C thawing regiment was the best treatment for E1 (Table 1), and though it was not significantly different from the other DMSO concentrations, it was used in E2 with some modifications. First, assuming a high tolerance of *R. marina* sperm, samples were incubated with DMSO 10% not only after thawing, but before freezing for two h at 4 °C, resulting in high embryo rates, close to control treatment (Table 2). This could indicate that sperm cells needed this amount of time before freezing to allow DMSO to enter the cells and protect them from cryoinjury, and before IVF to restore all their functionality after thawing osmotic stress (Hammerstedt et al. 1990).

Sperm concentration and frozen volume were also modified. A half testicle in two ml of solution in E1 resulted in 1.07; 1.25; and 0.99 × 10^7 sperm/ml for DMSO 5 %, 10 %, and 15 %, respectively. Half a testicle in 500 µl in E2 resulted in 3.41 and 3.23 × 10^7 sperm/ml for DMSO 10 % and DMSO 10 % + HEPES, respectively. Frozen volume in E1 and E2 were 250 µl and 100 µl, respectively. A smaller volume with higher sperm concentration might reduce the volume of water in the extracellular space, making less probable for ice

Table 1. Gastrula and abnormal embryo rates from E1 (n = 6 males).

Treatment	Gastrula rate		Abnormal embryo rate
	(M ± SD %)	Subgroups*	(M ± SD %)
Control	91.28 ± 7.58	a	-
DMSO 5% - RT	03.26 ± 4.00	b	-
DMSO 5% - 4C	19.48 ± 21.73	b	10.99 ± 2.98
DMSO 10% - RT	10.73 ± 13.00	b	-
DMSO 10% - 4C	23.17 ± 27.13	b	10.43 ± 4.64
DMSO 15% - RT	02.44 ± 3.13	b	-
DMSO 15% - 4C	07.90 ± 8.96	b	18.52 ± 10.76

M = mean, SD = standard deviation, RT = Room temperature thawing, 4C = 4 °C thawing.

*Subgroups by DMSO factor ($p < 0.001$, df = 15, $F = 93.97$) from two factor ANOVA.

to form during the time that the system reaches equilibrium at -80 °C. A reduction in ice nucleation avoids intracellular ice formation, and sperm lesions by ice crystals or hyperosmotic stress during freezing and/or thawing (Rubinsky 2003), thus contributing to protect sperm fertilizing capacity in E2. Sperm lysis can be inferred by the decreased post thawing sperm concentration in E2 (Table 2), but percentage of viable sperm cannot be determined because of the absence of membrane integrity or motility evaluation.

Experiment one (Table 1) showed significant differences in gastrula rates by CPA factor only between control and all DMSO treatments ($p < 0.001$, df = 15, F = 93.97). There were significant differences in gastrula rates for thawing factor, with 4 ºC thawing better than RT ($p < 0.001$, df = 15, $F = 20.94$). No interaction was found between CPA and thawing factors. Gastrula rates for DMSO concentrations at 4 °C were 19%, 23%, and 7% for DMSO 5%, 10%, and 15%, respectively. While gastrula rates for RT thawing were 3%, 10%, and 2% for DMSO 5%, 10%, and 15%, respectively (Table 1).

It is interesting that a slow thawing at 4°C had a higher gastrula rate than RT thawing considering that fast thawing is recommended to avoid recrystallization or osmotic injuries due to a prolonged exposure to the hyposmotic medium generated during melting (Rubinsky 2003) thus, anuran cryopreservation protocols use thawing temperatures of 21 °C and 30 °C (Browne et al. 1998; Sargent and Mohun 2005). Besides, a prolonged CPA exposure can be considered toxic (Fuller 2004), but in this case, samples used two h later gave higher gastrula rates than samples used immediately. Moreover, tail bud stage was reached by embryos of all DMSO treatments. These gastrula rates could indicate a high tolerance of *R. marina* sperm to prolonged DMSO exposure, as seen for other species like *Rana temporaria* which had been exposed to DMSO for 60 minutes with no detrimental effects on viability or motility (Mansour et al. 2010). Whether it was the temperature or the incubation time that led to higher gastrula rates reached by 4 °C thawing remains to be clarified.

In E2, cleavage rates (Table 2) were 97%, 75%, and 70% for Control, DMSO 10%, and DMSO 10% + H, respectively. Wilcoxon test found no significant differences between Control and DMSO 10% (z = -1.78, p = 0.075), nor between DMSO 10% and DMSO 10% + H (z = -0.52, p = 0.6); but there were significant differences between Control and DMSO 10% + H (z = -2.20, p = 0.028). There was an embryo reduction from second cleavage to tail bud stage in all treatments to 82%, 60%, and 54% tail bud embryos for Control, DMSO 10% and DMSO 10% + H, respectively (Table 2).

Since there were only three ovulating females used in this study, maternal effects could have influenced fertilization rates, so egg condition was revised before IVF. As expected from collection in the same locality during rainy season, only stage VI eggs were found in the oviducts of all females, indicating that they were in a similar reproductive status and the capability of eggs to be fertilized (Rastogi et al. 2011). Oogenetic stage VI is determinant for embryonic development because well differentiated animal and vegetal poles, a maximum size, and a postvitellogenetic condition indicate that oocytes are ready for ovulation (Dumont 1972). Ovulation in these females resulted in high gastrula and cleavage rates in control treatments from E1 (91%) and E2 (97%), both reaching tailbud stage.

Embryo developmental period in cryopreserved sperm treatments from E1 and E2 did not differ with the control treatments; all embryos developed in seven days from fertilization to tail bud stage. However, some abnormalities in tail bud stage were found in all treatments from E1, 4 °C thawing with DMSO 5%, 10%, and 15 % had 11%, 10%, and 18% abnormal embryos (Table 1). There is a 15% embryo reduction from second cleavage to tail bud stages in all treatments from E2. Apparently, it is not unexpected in natural frog populations to exhibit 2% abnormal embryos. Possible causes might be environ-

Table 2. Sperm concentration, cleavage and tail bud rates in control, DMSO 10%, and DMSO 10% + HEPES 2.5 mM treatments from E2 (n = 6 males).

	PF	PT	Cleavage rate		Tail bud rate
	(M ± SD x 10^7 sperm/ml)	(M ± SD x 10^7 sperm/ml)	(M ± SD %)	Subgroups*	(M ± SD %)
Control	2.50 ± 1.26	-	97.38 ± 01.84	a	82.74 ± 8.12
DMSO 10%	3.41 ± 2.38	1.78 ± 1.42	75.67 ± 25.22	a, b	59.99 ± 23.21
DMSO 10% + H	3.23 ± 2.06	1.28 ± 0.93	70.35 ± 19.74	b	54.46 ± 21.14

PF = Pre-freezing sperm concentration, PT = Post-thawing sperm concentration, M = mean, SD = standard deviation, H = HEPES 2.5 mM. *Subgroups from Wilcoxon test.

mental factors, such as UV radiation, extremes in pH, or thermal variations (Pašková et al. 2011). Higher percentages of abnormal embryos (60 %) can be possibly caused by xenobiotics, which interfere with embryo mechanisms for reactive oxygen species (ROS) regulation (Pašková et al. 2011). Captivity rearing conditions could cause ROS regulation to fail, with the consequential embryo abnormalities and mortality seen in E1 and E2, respectively. The presence of higher abnormal embryo percentages in captivity should be considered when planning to perform IVF for captive propagation.

We considered that HEPES could help to protect sperm functionality being one of Good's buffer qualities maintaining adequate pH values in culture media and has been used successfully in mammalian sperm cryopreservation (Will et al. 2011). Moreover, it has been used in a chemotaxis experiment with *Xenopus laevis* sperm (Al-Anzi and Chandler 1998) and we found it to retain sperm motility after a 48 h period at RT and 4 °C (unpublished data). But no improvement in cleavage or tail bud rates were found by the addition of this reactive to cryopreservation solutions (Table 2). The effect of HEPES on the cryopreservation of *R. marina* sperm remains unclear, though, it seems to be unnecessary.

The reported embryo rates in the present study suggest that frozen volume, sperm concentration, and DMSO incubation time can be key elements in improving embryo rates from IVF with cryopreserved sperm. *Rhinella marina* sperm seems to tolerate prolonged DMSO exposures at 4 °C, with favorable effects on sperm response to freezing and thawing. Nevertheless, freezing rates and cell viability or motility tests should be conducted to make possible stronger conclusions about the present data. We hope that this report leads to in-depth studies that can be applied to the conservation of more Neotropical species using ART.

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Belén Proaño graduated in Biological Sciences from Pontificia Universidad Católica del Ecuador (PUCE) in 2013. As an associated researcher at PUCE for two years, her investigations focused on reproductive biology and the application of Assisted Reproductive Technologies in Ecuadorian anurans under captivity conditions. Currently, she is working on personal projects away from the scientific environment, but with the same interest in understanding the wonder of life.

Oscar Pérez was born in Quito Ecuador. He obtained a doctoral degree in 2008 from Pontificia Universidad del Ecuador in collaboration with Duquesne University, Pennsylvania, USA. His advisors were Dr. Richard Elinson and Dr. Eugenia del Pino. Dr. Pérez is interested in the evolutionary comparison of development and the reproductive biology of Ecuadorian vertebrates. His current research focus is in finding new alternative models in developmental biology using the great Ecuadorian mega-diversity country as his playground. More particularly, his interest is in frog oogenesis—oocyte organization can vary between species and these variations can modify the developing pathway of the future embryo. Comparative methodologies are applied to find variations in oogenesis patterns in order to understand how these variations can modify embryogenesis features. These analyses employ a diversity of techniques such as histology, immunohistochemistry, genetic cloning, and bioinformatics tools in order to identify genes of importance for oogenesis and embryogenesis. All these efforts are focused towards shedding light on the reproduction and preservation of Ecuadorian fauna and its unique development features.

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Description of two new species similar to *Anolis insignis* (Squamata: Iguanidae) and resurrection of *Anolis* (*Diaphoranolis*) *brooksi*

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***Abstract.*—The spectacular giant anole lizard *Anolis insignis* is widely distributed but infrequently collected outside of northern Costa Rica. We recently collected several individuals similar to *Anolis insignis* from localities in Panama and southern Costa Rica. These populations differ from type locality *A. insignis* in male dewlap color and morphology. We associate one set of these populations with *Anolis* (*Diaphoranolis*) *brooksi* Barbour from Darién, Panama, and describe two additional populations as new species.**

Keywords. Central America, Costa Rica, lizard, Panama, Reptilia, taxonomy

Citation: Poe S and Ryan MJ. 2017. Description of two new species similar to *Anolis insignis* (Squamata: Iguanidae) and resurrection of *Anolis* (*Diaphoranolis*) *brooksi*. *Amphibian & Reptile Conservation* 11(2) [General Section]: 1–16 (e141).

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Introduction

Costa Rica and Panama contain perhaps the most studied herpetofauna of the Neotropics for ecology and systematics (Savage 2002; Donnelly et al. 2005). The early works of Taylor (e.g., 1956) and then Savage (e.g., 1975), along with the development of the Organization for Tropical Studies (OTS) and the efforts of the University of Costa Rica (UCR), have established Costa Rica as a center of herpetological research. The Smithsonian Tropical Research Institute (STRI) has been instrumental in fostering herpetological work in Panama.

The *Anolis* lizards of Costa Rica and Panama are well studied (Taylor 1956; Savage 2002), but new species continue to be discovered (e.g., Kohler 2011; Poe et al. 2015). As of 28 February 2016 the Reptile Database lists 42 species of *Anolis* from Costa Rica and 45 species from Panama. Relatively unexplored regions such as the southern Cordillera de Talamanca in Costa Rica and the Darién Region of eastern Panama are likely to produce new discoveries, and detailed molecular studies such as those undertaken in frogs (Crawford et al. 2010) are likely to unearth cryptic diversity of *Anolis*.

We have conducted extensive fieldwork on *Anolis* in Costa Rica and Panama since 2006. During this time, we have collected numerous individuals of *Anolis* that might standardly be assigned to the spectacular and rarely collected giant anole species *A. insignis* (Fig. 1). We have noticed numerous differences between populations of this species that are consistent within geographically distinct populations. We now possess enough material to confidently distinguish and recognize three species of *Anolis* similar to *A. insignis*. Herein we resurrect a previously synonymized name and describe two new species.

Materials and Methods

We adopt the evolutionary species concept (Simpson 1961; Wiley 1978) and operationalize this concept by identifying species based on traits that are consistent within hypothesized species but differ among species.

Measurements were made with digital calipers on preserved specimens and are given in millimeters (mm), usually to the nearest 0.1 mm. Specimens are referenced from the Museum of Southwestern Biology (MSB), the Museum of Comparative Zoology (MCZ), the Los Angeles County Museum (LACM), the Museo de Vertebrados, University of Panama (MVUP), and the University of Costa Rica (UCR). Snout–vent length (SVL) was measured from tip of snout to anterior margin of the cloaca. Head length was measured from tip of snout to anterior margin of the ear opening. Head width was measured at

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Fig. 1. *Anolis insignis*, male, Pocosol, Alajuela, Costa Rica.

the broadest part of the head, between the posterolateral corners of the orbits. Femoral length was measured perpendicularly from the longitudinal midline of the venter to the knee, with limb bent at a 90° angle. Terminology and characters for qualitative conditions and scale counts follow standards established by Ernest Williams (e.g., Williams et al. 1995).

We tested for the objective identification of hypothesized groups (i.e., species) using the Multiresponse Permutation Procedure (MRPP; Mielke 1984) as described by McCune and Grace (2002). Like the commonly-used discriminant function analysis (DFA), MRPP is among the class of techniques used to test for the distinctiveness of a-priori hypothesized groups. We use this test rather than DFA because we are not confident making distributional assumptions about our data and we suspect the nonparametric nature of this approach will treat our small sample sizes more conservatively. We hypothesized groups based on male dewlap color pattern and geography (see below) and employed the following characters: number of lamellae on 4th toe (counted in the manner of Williams et al. [1995]), number of postmental scales, number of postrostral scales, number of scales across the snout at the second canthals, number of supralabial scales to the center of the eye, number of scales between the supraorbital semicircles, number of scales from the interparietal to the supraorbital semicircles, number of loreal rows. As none of these traits are the basis for our diagnoses (see below), this analysis provides a somewhat independent check of our species inferences. We used Euclidean distances of standardized data (i.e., mean = 0, standard deviation = 1) and present observed and expected Delta (i.e., the test statistic), *P*-value based on 99 randomizations, and Chance Corrected Within Group Agreement (i.e., effect size). Sexual dimorphism, if present, appeared to be less than interspecific dimorphism for the studied traits. Therefore to increase our small sample sizes we analyzed both sexes together. We demonstrate this lack of clustering by sex in two ways. First, we performed the same MRPP analysis but grouped by sex. Second, we performed Principal Component Analysis (PCA) of the above characters and present bivariate graphs of the first two principal components labeled by sex and by hypothesized species. Although PCA may not be appropriate for statistical interpretations and tests given our small sample sizes and high observation-to-variable ratio (see below; although we note that similar PCA results are obtained with subsamples of variables), we believe this technique nevertheless to be useful for the limited purpose of visualizing gross differences in clustering patterns by sex versus by species. Statistical analyses were performed in Stata (2013) and Microsoft Excel.

The hypothesized new species were found to form a well-supported clade with *Anolis insignis*, *A. microtus*, and *A. ginaelisae* (Bayesian Posterior Probability of 100%) by Poe et al. (2015), who included all known *Dactyloa*-clade *Anolis* in their phylogenetic analysis. Terminal taxa NSP.E, NSP.F, NSP.L in Poe et al.'s (2015) Fig. 5 correspond to species described herein. In order to more finely examine the interrelationships of the *insignis*-like anoles, we added new morphological data to the data matrices of Poe et al. (2015) and Poe et al. (2017), and analyzed these data for *A. insignis*, *A. microtus*, *A. ginaelisae*, the three additional species described here, and two *Dactyloa*-clade outgroups (*A. frenatus*, *A. fraseri*). We eliminated characters that did not vary in the ingroup and added characters based on our examination of specimens for the current study. The final matrix includes 18 characters of morphology and 50 genes of DNA sequence data. Additional details of data properties and collection (i.e., gene names, data sources, partitioning) are in Poe et al. (2017). Morphological characters were rescaled differently from Poe et al. (2017) to account for new data and our restricted taxon sample.

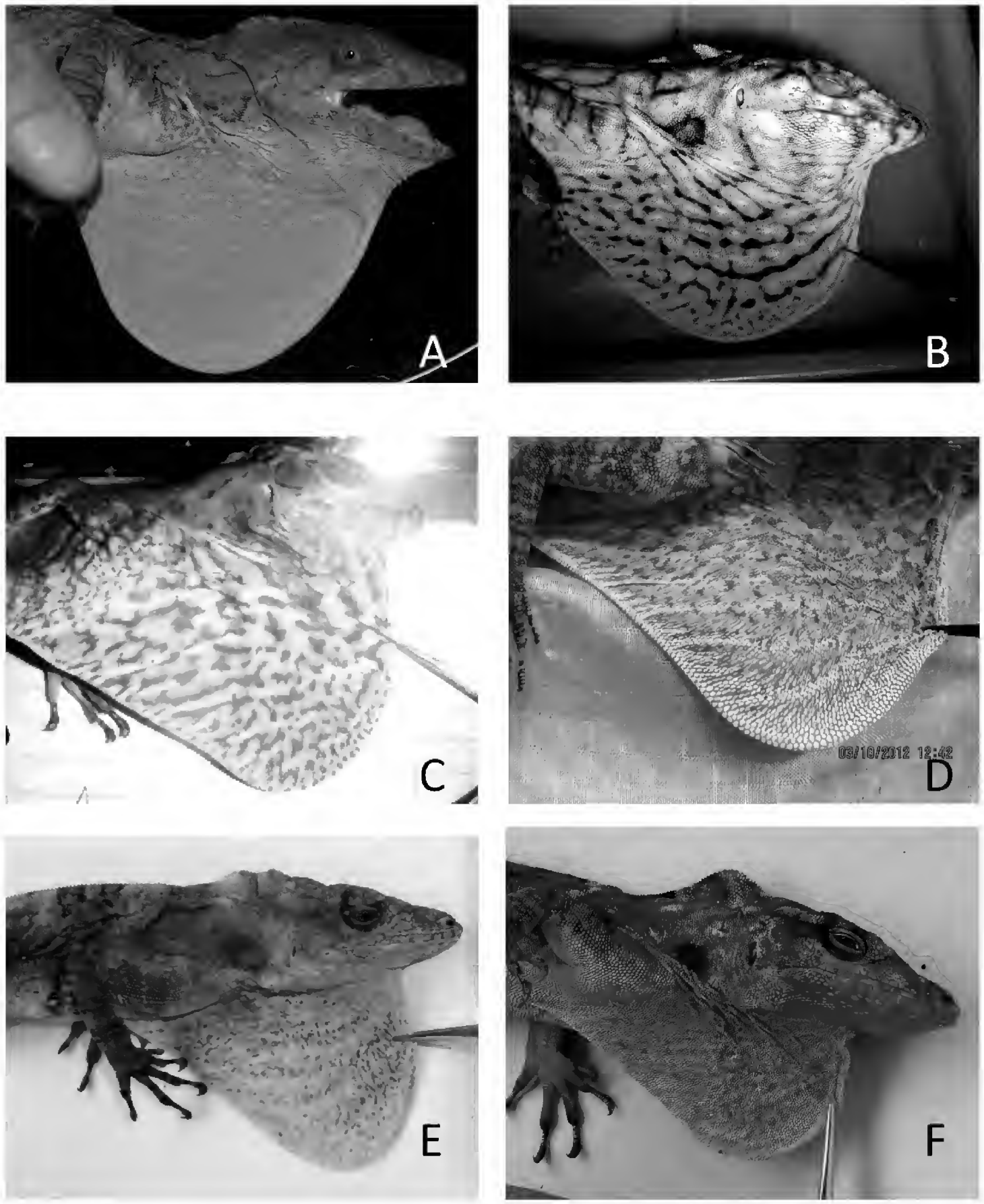

Fig. 2. Dewlaps of **A)** *Anolis brooksi*, male, El Copé, Panama; **B)** *A. brooksi*, female, El Copé, Panama; **C)** *A. savagei*, male, Las Cruces, Costa Rica; **D)** *A. savagei*, female, Las Cruces, Costa Rica; **E)** *A. kathydayae*, male, Fortuna, Panama; **F)** *A. kathydayae*, female, Fortuna, Panama.

Although this data matrix includes 24,897 characters, we note that only the morphological dataset is informative for the interrelationships of *A. insignis* and the other three species discussed in depth in this paper, as only two of the discussed species are scored for some DNA sequence data. The included DNA data are useful for establishing the monophyly of these forms with *A. microtus* and *A. ginaelisae* and examining genetic divergences as they relate to hypothesized species (see below). The phylogenetic matrix analyzed for this paper is available electronically at: stevenpoe.net. The morphological characters and data matrix are in Appendices 1 and 2 respectively.

We analyzed this matrix using a Bayesian phylogenetic approach as implemented in MrBayes (Huelsenbeck and Ronquist 2001) using the model parameters and settings of Poe et al. (2017), except that a heating temperature of 0.01 was used and the analysis was carried out for 2,000,000 generations. That is, we included separate GTR + G models for each of 15 DNA partitions of the 50 genes (including partitions by codon position for the best-sampled protein coding genes COI and ND2) with partitions determined by Partitionfinder (Lanfear et al. 2012) and model-averaging across the entire GTR model space for each gene partition ("nst=mixed" in MrBayes). Morphological character evolution was modeled with the "standard" MrBayes model. We checked for convergence of parameter values by examining estimated sample sizes in Tracer (Rambaut et al. 2014).

Results

Four very different male dewlap types are recognizable (Figs. 1, 2) and correlate with geography. Male specimens from central and northern Costa Rica have orange-

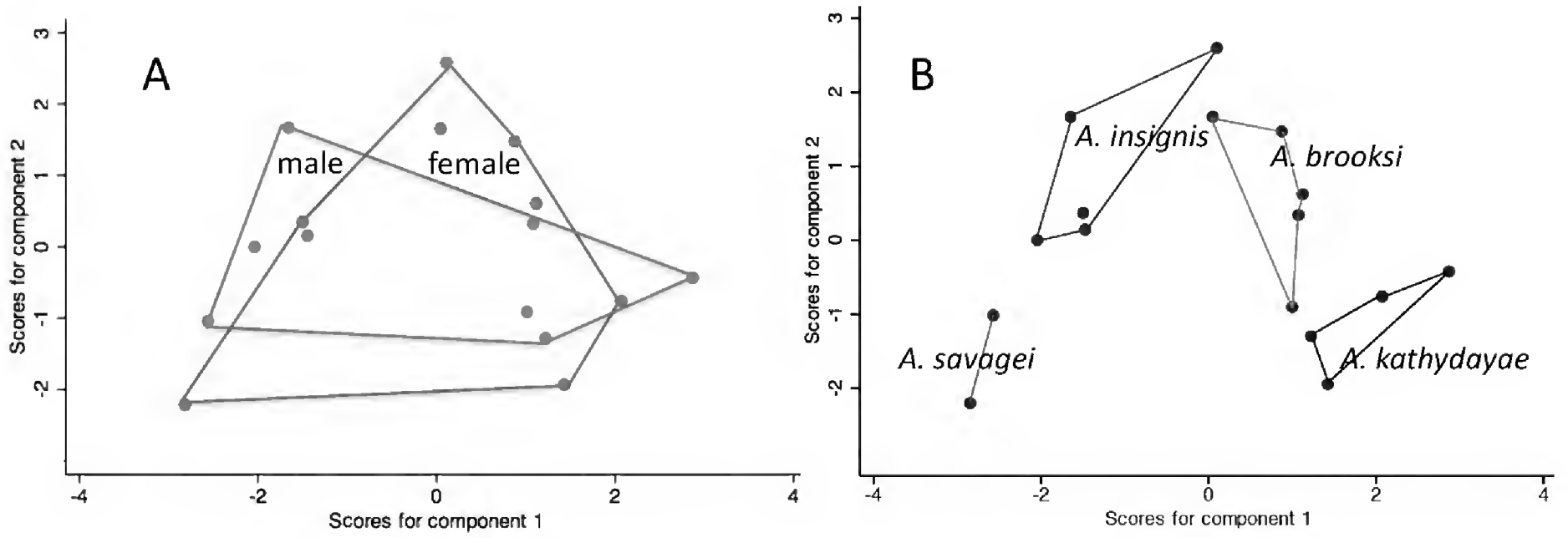

Fig. 3. Graph of principal components 1 and 2 for traits used in MRPP analysis of species of *Anolis* studied here, labeled by **A)** sex and **B)** putative species.

red dewlaps; those from southwestern Costa Rica have pale pink dewlaps with black streaks; those from the Fortuna area in Panama have white dewlaps; and those from eastern Panama (Santa Fé, El Copé, Cerro Azul) have peach-tan dewlaps. We hypothesize that these differences represent inter- rather than intraspecific variation for four reasons. First, we observed at least three adult males within each range, with no significant variation in male dewlap color pattern at any locality or between localities where a particular dewlap type was found. Second, the degree of difference among these male dewlap color patterns would be unprecedented as intraspecific variation in *Anolis*. Third, each male dewlap-group is distinguishable by additional traits (see below). Fourth, groups identified by male dewlap color are different according to MRPP. The MRPP analysis was significant ($P = 0.01$, 99 randomizations; Delta = 3.09, Deltanull = 3.85), rejecting the null hypothesis of random assignment of individuals to groups. The Chance Corrected Within Group Agreement was 0.20, indicating 20% within group agreement above that expected by chance. The MRPP analysis was nonsignificant when individuals were grouped by sex rather than by hypothesized species ($P = 0.24$, 99 randomizations; Delta = 3.75; Deltanull = 3.85), which is compatible with our observation of a lack of sexual dimorphism in these characters. Figure 3 shows that our studied individuals do not cluster morphologically by sex according to PCA of traits used in the MRPP. Based on this evidence, we are comfortable pooling our samples by sex within species for the MRPP analysis.

We associate the Costa Rican specimens examined from near the city of San José with the nominate species *Anolis insignis* Cope 1871 (Type locality: "San José"). Our central Panama specimens from Cerro Azul, Panamá province, and El Copé, Coclé province may be an unrecognized lineage. Alternatively, on geographic and morphologic grounds they may be associated with *Diaphoranolis brooksi* Barbour (holotype MCZ 16297) from the Darién of Panama—an individual previously determined to be "an unquestioned juvenile of *A. insignis*" (Savage and Talbot 1978). As a preserved specimen, the *A.* (= *Diaphoranolis*) *brooksi* holotype specimen appears similar to juveniles we collected at El Copé, and we lack adult dewlap photos and adult specimens for the Darién population. We choose to assign our easternmost form to *A. brooksi* pending future collection of *A. insignis*-like anoles in Darién. The distinctive populations from Fortuna, Chiriquí, Panama, and Las Cruces, Puntarenas, Costa Rica, currently lack names.

Below we redescribe *Anolis insignis* from specimens near the city of San José Costa Rica, and *A. brooksi* from specimens from El Copé and Cerro Azul in Panama. We describe two new species from Las Cruces, Costa Rica, and Fortuna, Panama respectively. We describe variation in *A. insignis* and *A. brooksi* and describe holotype specimens for the two new species. Comparisons among the four species are summarized in Table 1. The results of our phylogenetic analysis of these species are summarized in Fig. 4. We infer that the Markov Chain Monte Carlo analysis was run long enough to sample parameters in proportion to their true posterior probability distributions based on low standard deviation of split frequencies (0.011) and estimated sample sizes well above 200 for all parameters, as recommended by Rambaut et al. (2014).

Systematics

Anolis insignis Cope 1871

(Figures 1, 5)

Holotype

Lost (Savage and Talbot 1978); from "Costa Rica: Provincia de San José: near Ciudad San José; probably from near La Palma," according to Savage and Talbot (1978) and Savage (1974).

Examined specimens

LACM 149495 collected by J. Hagnauer and N.J. Scott in January 1975 (no day provided) and LACM 149496 collected by G. Hagnauer and W. Timmerman in April 1974 (no day provided) from Costa Rica, Alajuela, Vicinity of Bijagua (10.7333; -85.1; 425 m); LACM 149500

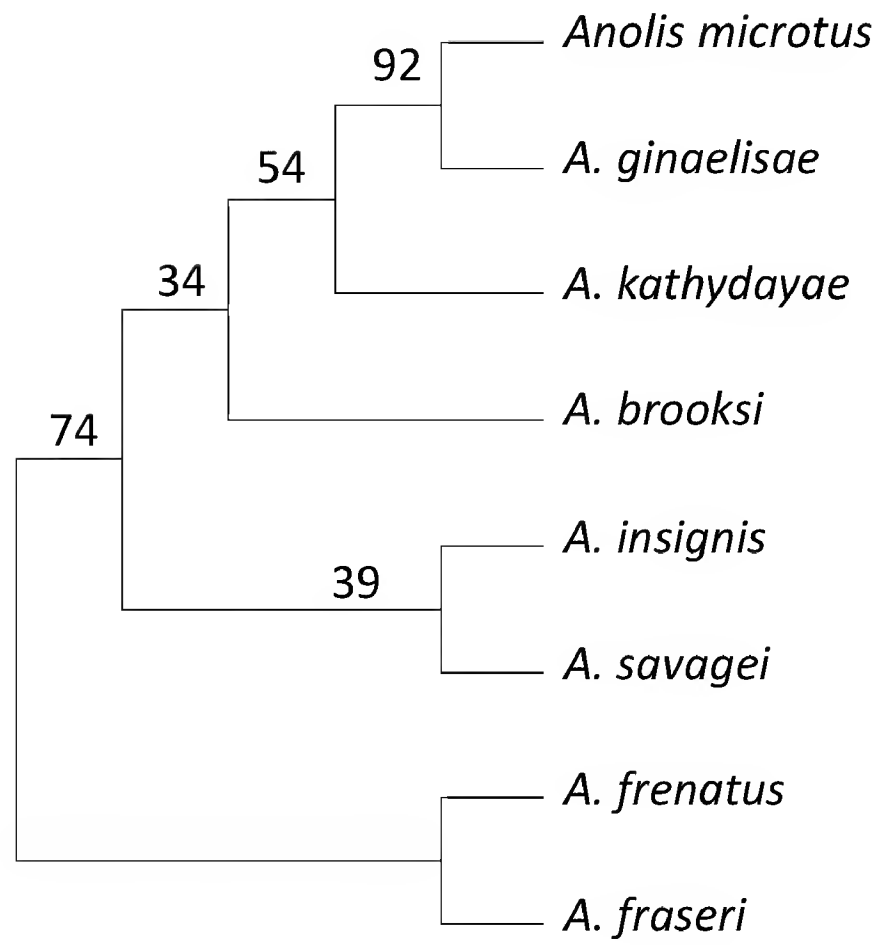

Fig. 4. Bayesian phylogenetic estimate of relationships for *Anolis* species similar to *A. insignis*. Numbers on clades are posterior probabilities.

collected by K. Timmerman 20 June 1984 and LACM 149497 collected by H. Hespenheide and E. Fisher (no date provided) from Costa Rica, Puntarenas, Monteverde (10.3; -84.816667; 1,455 m); LACM 149498 collected by P. Siegfried (no date provided) from Costa Rica, Alajuela, Poco Sol (10.3667; -84.6167; 580 m).

Diagnosis

Anolis insignis and the three species described below are the only Central American *Anolis* to combine large size (> 120.0 mm SVL), smooth scales on the upper thigh, and short limbs (Savage and Talbot 1978). *Anolis insignis* is diagnosed from the three species described below by its orange-red male dewlap (Fig. 1; white, peach-tan, and pink with dark streaks, respectively by species, in the other forms). It further differs from the Southwestern Costa Rican form in its lack of a postorbital blotch (present in the Southwestern Costa Rican form); from the Fortuna form in its prominent postcloacal scales in males (obscure in the Fortuna form); from *A. brooksi* in some scale counts (Table 1; e.g., greater number of postrostrals) and details of color pattern (Savage and Talbot 1978; e.g., absence of narrow black lines dorsally).

External description (in mm)

Snout-vent length (SVL) to 157.0 mm male, 140.0 mm female; head length-SVL ratio 0.24–0.25, head width-SVL ratio 0.14–0.16; ear height-SVL ratio 0.015–0.028; femoral length-SVL ratio 0.24–0.25; tail length-SVL ratio 1.9–2.1. Dorsal head scales mostly smooth, a few with weak keels or rugosity apparently reflecting underlying bone or ossification, pustules present in some specimens; frontal depression present, anterior half of snout raised in two faint parallel rows; rostral overlaps mental anteriorly; lateral edges of mental scales extend farther posteriorly than rostral; 9–11 scales across snout between second canthals; 2–3 scales between supraorbital semicircles; 2–3 scales separating interparietal and supraorbital semicircles; suboculars in contact with supralabials; five loreal rows; no elongate superciliaries, first superciliary is smaller than first canthal; anterior row of small scales following canthals along edge of orbit; circumnasal scale separated from rostral by one scale; interpari-

Table 1. Morphological traits of species similar to *Anolis insignis*. Measurements are in millimeters. Means are given with ranges in parentheses. Measurement characters were scored only for adults.

	Anolis insignis	***A. brooksi***	***A. kathydayae***	***A. savagei***
	n = 2 males, 3 females	*n* = 3 males, 2 females	*n* = 2 males, 2 females	*n* = 1 male, 1 female
Snout to vent length male	154.5 (152.0–157.0)	152.7 (129.5–176.0)	142.3 (136.6–148.0)	141.1 (141.1)
Snout to vent length female	139.0 (138.0–140.0)	134.0 (134.0)	136.1 (136.1)	(juvenile)
Head length male	37.4 (36.2–38.6)	36.0 (30.5–41.4)	36.4 (34.8–38)	33.0 (33.0)
Head length female	34.6 (33.6–35.3)	34.8 (34.8)	33.9 (33.9)	–
Head width male	21.6 (20.8–22.4)	21.4 (18.1–24.7)	21.6 (20.7–22.5)	21.0 (21.0)
Head width female	21.3 (20.4–22.6)	20.8 (20.8)	21.2 (21.2)	–
Ear height male	3.0 (2.3–3.6)	3.7 (3.4–4.1)	4.2 (3.9–4.5)	2.9 (2.9)
Ear height female	3.5 (2.9–3.9)	3.7 (3.7)	4.1 (4.1)	–

Table 1 (continued). Morphological traits of species similar to *Anolis insignis*. Measurements are in millimeters. Means are given with ranges in parentheses. Measurement characters were scored only for adults.

	Anolis insignis	***A. brooksi***	***A. kathydayae***	***A. savagei***
	n = 2 males, 3 females	*n* = 3 males, 2 females	*n* = 2 males, 2 females	*n* = 1 male, 1 female
Femoral length male	37.9	37.5	36.3	30.4
	(36.7–39.1)	(31.6–43.4)	(34.2–38.5)	(30.4)
Femoral length female	34.5	33.4	32.7	–
	(33.5–35.1)	(33.4)	(32.7)	
4th toe length male	25.2	21.4	22.4	19.9
	(24.7–25.7)	(19.1–23.7)	(20–24.8)	(19.9)
4th toe length female	23.4	21.5	20.2	–
	(22.4–24.7)	(21.5)	(20.2)	
Tail length	294.0	291.6	284.0	245.0
	(287.0–310.0)	(240.0–355.0)	(275.0–292.0)	(245.0)
Number of dorsal scales in 5% SVL	9.5	11.6	9.0	8.0
	(7–11)	(11.0–12.0)	(9.0)	(8.0)
Number of ventral scales in 5% SVL	9.5	8.5	10.0	8.0
	(8.0–11.5)	(8–9)	(10.0)	(8.0)
Number of scales across snout at second canthals	10.0 (9.0–11.0)	10.4 (10–11)	10.0 (9.0–11.0)	8.5 (8.0–9.0)
Number of scales between supraorbital semicircles	2.2 (2.0–3.0)	3.4 (3.0–4.0)	3.2 (3.0–4.0)	2.0 (2.0)
Number of scales between interparietal and supraorbital semicircles	2.6 (2.0–3.0)	3.0 (2.0–4.0)	3.2 (3.0–4.0)	1.5 (1.0–2.0)
Number of postrostral scales	7.8	6.8	5.7	6.5
	(7.0–10.0)	(6.0–7.0)	(5.0–6.0)	(6.0–7.0)
Number of postmental scales	7.4	6.0	5.0	7.5
	(6.0–9.0)	(5.0–7.0)	(4.0–5.0)	(7.0–8.0)
Number of scale rows separating suboculars and supralabials	0	0	0	0
Number of supralabials from rostral to center of eye	8.2 (8.0–9.0)	8.0 (7.0–9.0)	7.2 (7.0–8.0)	7.0 (7.0)
Number of lamellae under phalanges II & III of 4th toe	26.6 (25.0–27.0)	26.4 (25.5–27.5)	25.5 (23.5–27.0)	26.7 (25.0–28.5)
Number of loreal rows	5.0	5.4	6.0	4.5
	(5.0)	(5.0–6.0)	(6.0)	(4.0–5.0)
Posterolateral extent of mental	<= rostral	>= rostral	<=rostral	<rostral

etal length-SVL ratio 0.014–0.017; 8–9 supralabials to center of eye; 6–9 postmentals; 7–10 postrostrals; scales in supraocular disc only slightly differing in size; mental partially divided posteriorly, extending posterolaterally equal to or shorter than rostral, with straight posterior border; 0–2 keeled enlarged sublabials.

Dewlap reaches well posterior to axillae in males and females; dewlap scales in rows of multiple scales in both sexes; no axillary pocket; pair of distinct, abruptly enlarged postcloacal scales in males; dorsal scales smooth; zero enlarged middorsal rows, 7–11 longitudinal rows in 5% of SVL; ventral scales in transverse rows, smooth, 8–12 scales in 5% of SVL; supradigitals multicarinate; toepads expanded; 25–27 lamellae under third and fourth phalanges of fourth toe; thigh scales smooth dorsally and ventrally, unicarinate anteriorly, multicarinate at knee; tail with a double row of middorsal scales.

Distribution and habitat

We have no experience with *Anolis insignis* in life. Savage (2002) reports that this is an uncommon canopy species that inhabits undisturbed forests.

With our recognition of multiple species within what was previously considered *Anolis insignis*, we restrict the range of *A. insignis* sensu stricto to the Cordillera Tilarán and Cordillera Central of Costa Rica. We currently consider the range of *A. insignis* to encompass localities for *A. insignis*-like anoles collected in Northern and Central Costa Rica. Assuming this range, the known elevation of *A. insignis* is 425 m (Bijagua, CRE 3715, UCR 8783) to

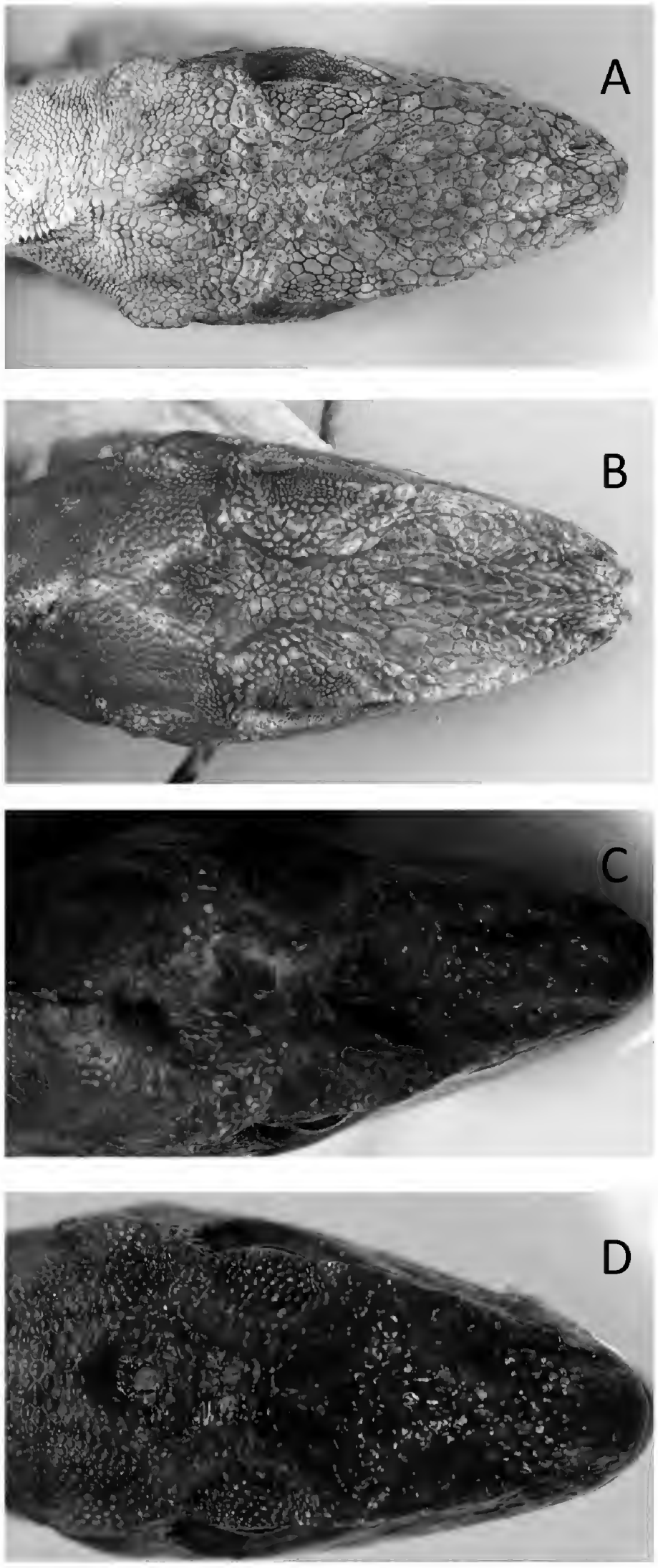

Fig. 5. Dorsal headscales of **A)** *Anolis kathydayae*, MSB 96613; **B)** *A. brooksi*, MSB 75647 **C)** *A. savagei*, MSB 96616; **D)** *A. insignis* LACM 149500.

1,500 m (La Palma, Holotype).

Anolis brooksi Barbour 1923

(Figures 2, 5–7)

Holotype

MCZ 16297 *Diaphoranolis brooksi*, juvenile female, from Mt. Sapo, Darién, Panama, 2,500 feet elevation;

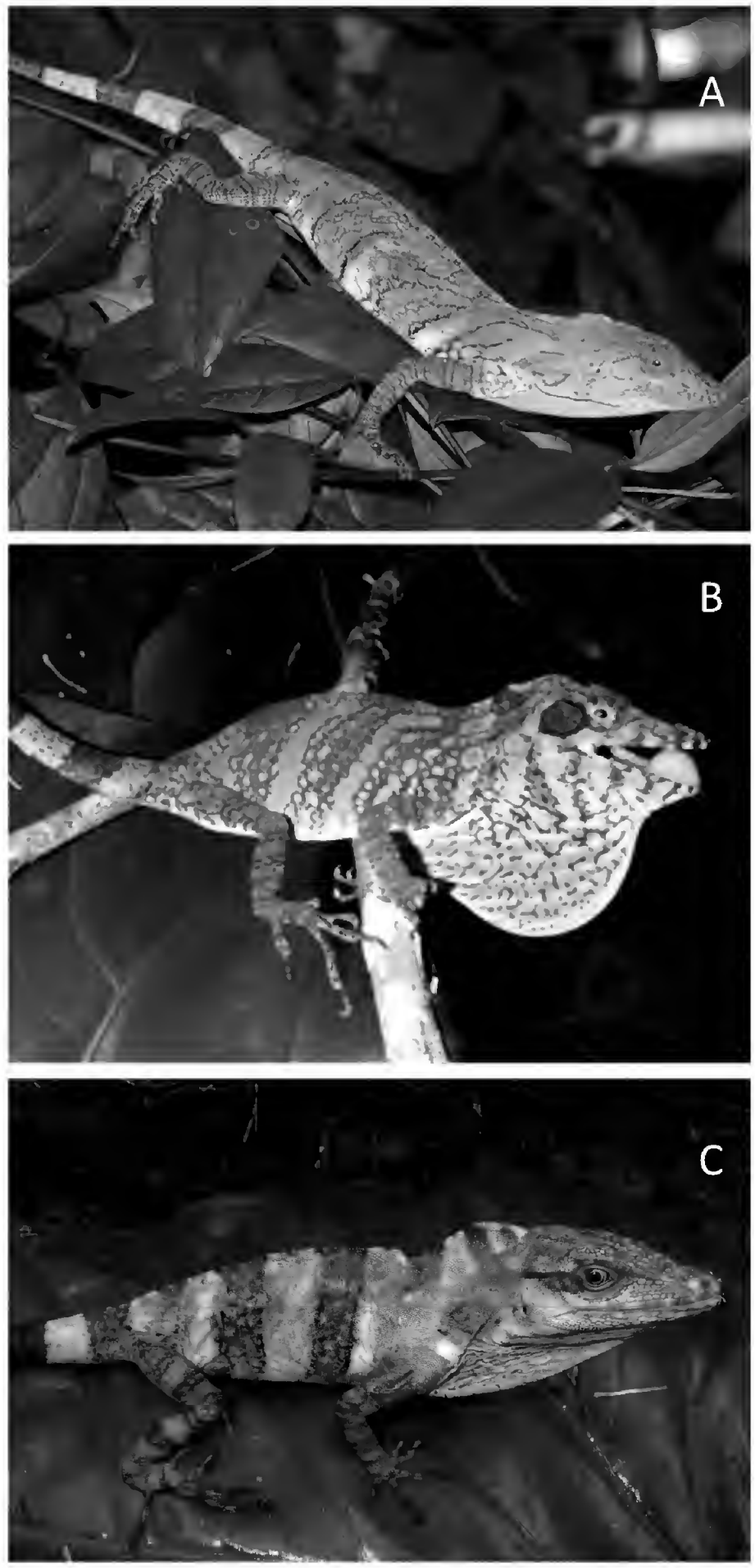

Fig. 6. Adult male individuals of **A)** *Anolis brooksi*, El Copé, Panama; **B)** *A. savagei*, Las Cruces, Costa Rica; **C)** *A. kathydayae*, Fortuna, Panama.

collected by Thomas Barbour and Winthrop Brooks, in April, 1922.

Examined specimens

Parque Nacional G.D. Omar Torrijos H., Coclé Province, Panama; 8.668, -80.593, 775 m: MSB 79924, MSB 79922, MSB 79923, MSB 75647, MSB 79925. Specimens examined but not scored for quantitative analysis: Cerro Azul, Panamá, Panama: MVUP 2007. Mt. Sapo, Darién, Panama: MCZ 16297 (holotype).

Diagnosis

Anolis insignis, *A. brooksi*, and the two species described below are the only Central American *Anolis* to combine

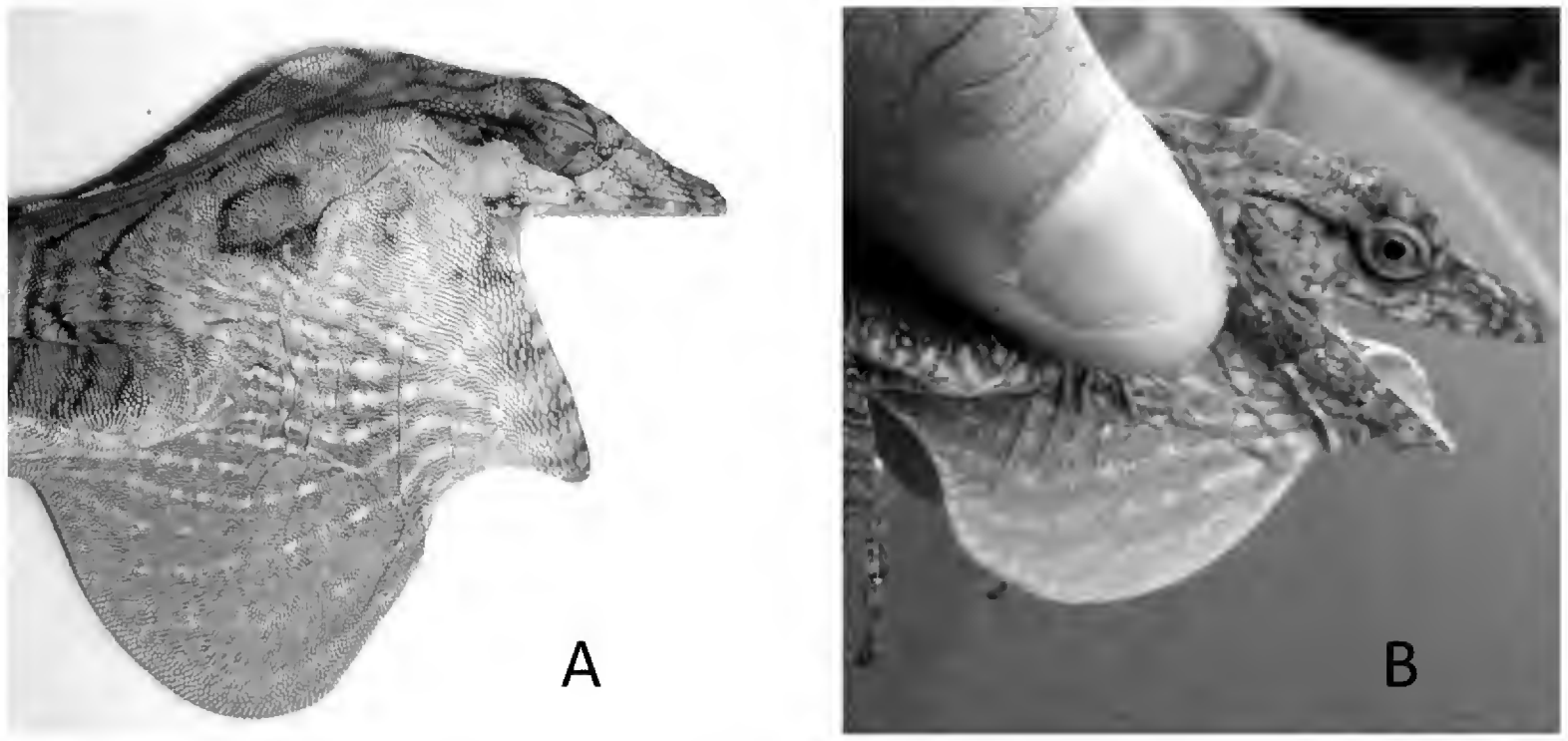

Fig. 7. Dewlaps of males of *Anolis brooksi* from **A)** Cerro Azul, Panama (MVUP 2007); **B)** Santa Fé, Panama (not collected).

large size (> 120.0 mm SVL), smooth scales on the upper thigh, and short limbs (Savage and Talbot 1978). *Anolis brooksi* is diagnosed from the three other *insignis*-like anole species discussed here by its peach-tan male dewlap (Fig. 2; orange-red in *A. insignis*; white, pale pink with dark streaks, respectively by species, in the other two forms). It further differs from the Southwestern Costa Rican form in its lack of a postorbital blotch (present in the Southwestern Costa Rican form) and its female dewlap color pattern (white or brown with dark streaks; pale pink with dark streaks in the Southwestern Costa Rica form); from the Fortuna form in its prominent postcloacal scales in males (obscure in the Fortuna form) and its female dewlap color pattern (white or brown with dark streaks; patternless white in the Fortuna form); from *A. insignis* in some scale counts (Table 1; e.g., fewer postrostrals) and details of color pattern (Savage and Talbot 1978; e.g., presences of narrow black lines dorsally).

Description (measurements in mm)

Snout–vent length to 176.0 mm male, 134.0 mm female; head length-SVL ratio 0.24–0.26, head width-SVL ratio 0.14–0.16; ear height-SVL ratio 0.023–0.028; femoral length-SVL ratio 0.24–0.25; tail length-SVL ratio 1.9–2.1. Dorsal head scales mostly smooth; frontal depression present, anterior half of snout raised in two faint parallel rows; rostral overlaps mental anteriorly; lateral edges of mental extend farther posteriorly than rostral; 10–11 scales across snout between second canthals; 3–4 scales between supraorbital semicircles; 2–4 scales separating interparietal and supraorbital semicircles; suboculars in contact with supralabials; 5–6 loreal rows; no elongate superciliaries, first superciliary is approximately equal in size to first canthal; row of small scales following canthals along edge of orbit; circumnasal scale separated from rostral by 1–2 scales; interparietal length-SVL ratio 0.014–0.015 (or absent); 7–9 supralabials to center of eye; 5–7 postmentals; 6–7 postrostrals; some enlarged scales present in supraocular disc (or all scales approximately equal), decreasing gradually in size; mental partially divided posteriorly, extending posterolaterally beyond rostral, with posterior border straight or in convex or concave arc; 1–2 keeled enlarged sublabials. Dewlap reaches well posterior to axillae in males and females; dewlap scales in rows of multiple scales in both sexes; no axillary pocket; distinct, abruptly enlarged postcloacal scales present in males; dorsal scales smooth; zero enlarged middorsal rows, 11–12 longitudinal rows in 5% of SVL; pair of middorsal scale rows raised in largest specimen; nuchal crest present with slightly enlarged triangular middorsal scales; ventral scales in transverse rows, smooth, 8–9 scales in 5% of SVL; supradigitals multicarinate; toepads expanded; 25–28 lamellae under third and fourth phalanges of fourth toe; thigh scales smooth dorsally and ventrally, unicarinate anteriorly and multicarinate at knee; tail with a double row of middorsal scales.

Color pattern in life

Adult males from El Copé (MSB 75647) and Cerro Azul (MVUP 2007) appeared mainly tan dorsally, with diffuse banding of white, black, green, peach, and dark brown. The limbs and digits were banded with narrow double lines of black or dark green. The tail was patterned with distinct black and greenish bands. The dewlap was solid peach-tan. An adult female (MSB 79925) appeared similar to the males but possessed scant green dorsally, with a white dewlap with prominent dark streaking. A dark shoulder blotch is evident in individuals in some of our photos of adults, but not in others. The iris is red. The throat is light and the tongue appeared peach in an El Copé specimen but yellow in the specimen from Cerro Azul. Males from Cerro Azul and Santa Fé had dewlaps similar to the El Copé specimen, but slightly paler (Fig. 7). An uncollected specimen from Isla Escudo de Veraguas, Bocas del Toro, that we tentatively allocate to this species had a dewlap similar to those figured here but with a brighter, slightly orange-yellow tint. An adult

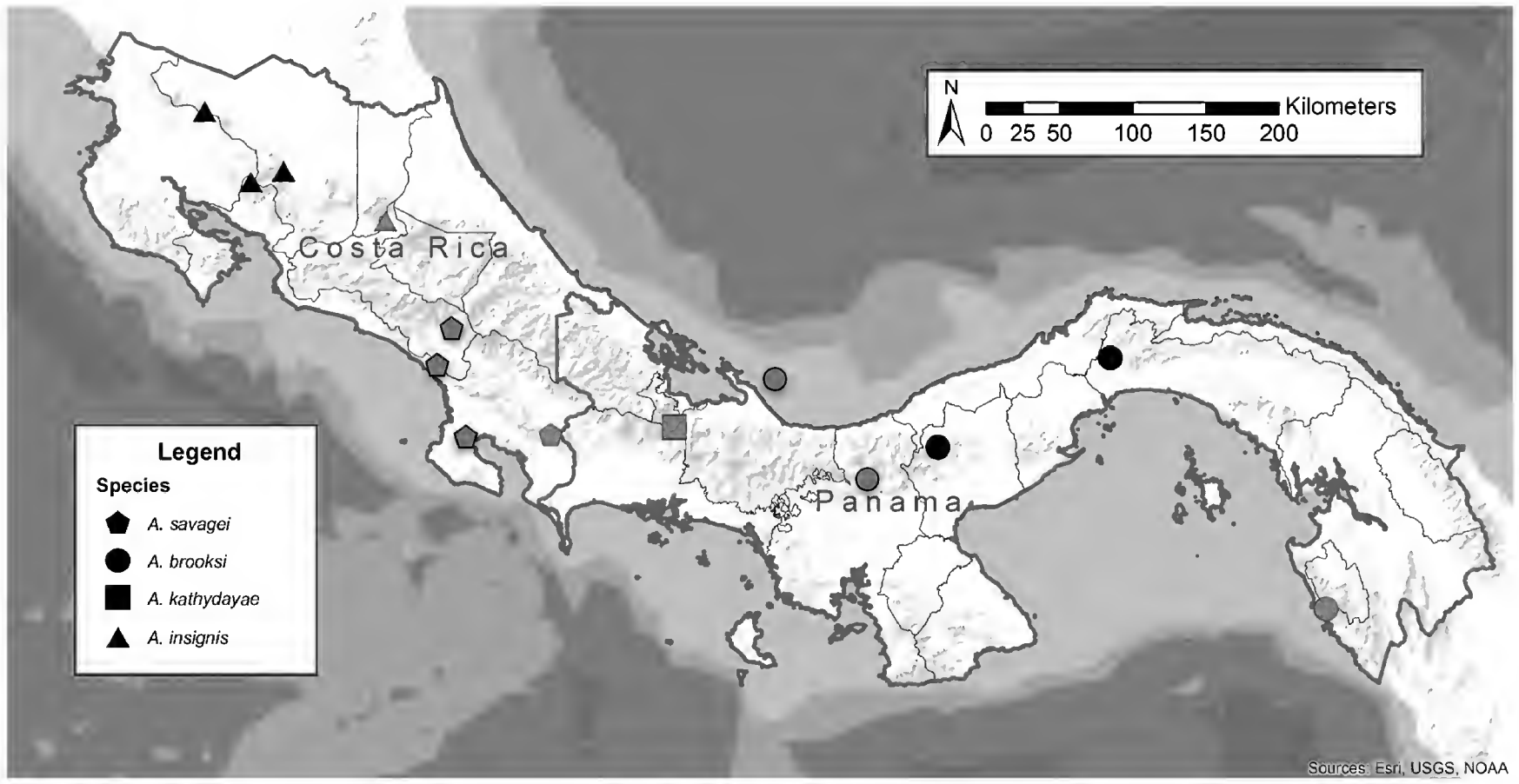

Fig. 8. Map of Panama and Costa Rica, showing localities for specimens referenced in text. Type localities are in red. Black symbols are specimens examined (type locality specimens also were examined for all species). Gray symbols represent unexamined specimens or photographic evidence discussed in text. Each point may represent multiple individuals (see text).

female dewlap figured by Lotzkat et al. (2013) was light brown with dark streaks.

Distribution and habitat

We collected *Anolis brooksi* in El Copé and Cerro Azul sleeping at night on saplings and tree branches from three to five meters above the ground. Specimens were collected in dense secondary forest (El Copé) and in disturbed habitat (Cerro Azul). Photographic evidence of male dewlap color pattern indicates the species is present at Santa Fé, Veraguas (see below) and, potentially, Isla de Escudo, Bocas del Toro (pers. obs.). Thus, *A. brooksi* appears to occur from sea level to 970 m from Darién north to Bocas del Toro.

Anolis savagei, new species

(Figures 2, 5, 6)

urn:lsid:zoobank.org:act:1F0F7528-F3D6-43B3-993D-E7AEBCB5A39C

Holotype

MSB 96616, adult male, collected at Las Cruces, Puntarenas, Costa Rica; 8.78242, -82.95886, 1,127 m; collected by Steven Poe, Eric Schaad, Ian Latella, and Mason Ryan on 20–23 March 2009.

Paratypes

UCR 20635 (not scored; POE 2671); LACM 149499 collected by R.W. McDiarmid on 21 Aug 1971 from Costa Rica, Puntarenas, San Vito de Java, OTS Las Cruces Biological Station (8.816667; -82.966667; 1,100 m).

Diagnosis

Anolis insignis, *A. brooksi*, *A. savagei*, and the species described below are the only Central American *Anolis* to combine large size (> 120.0 mm SVL), smooth scales on the upper thigh, and short limbs (Savage and Talbot 1978). *Anolis savagei* is distinguished from *A. insignis*, *A. brooksi*, and the form described below by its male dewlap color pattern of pale pink with dark streaks (orange-red in *A. insignis*; peach-tan in *A. brooksi*; white in the form described below; Figs. 1, 2) and presence of a prominent postorbital blotch (absent in *A. insignis*, *A. brooksi*, and the form described below).

Etymology

This name is a patronym to honor Dr. Jay M. Savage for his contributions to Neotropical herpetology, especially his seminal works, mentorship, and leadership in tropical biology and conservation in Costa Rica. Dr. Savage helped found the Organization of Tropical Studies (OTS) and the type locality of this species is the Las Cruces OTS field station.

Description of holotype

Snout-vent length 141.0 mm; head length-SVL ratio 0.23, head width-SVL ratio 0.15; ear height-SVL ratio 0.021; femoral length-SVL ratio 0.22; tail length-SVL ratio 1.74. Dorsal head scales smooth, some rugose; frontal depression present, dorsum with weak parallel rows evident anteriorly; rostral overlaps mental anteriorly; eight scales across snout between second canthals; two scales between supraorbital semicircles; one scale separating interparietal and supraorbital semicircles; subocular in contact with supralabials; five loreal rows; zero elongate superciliaries, first large scale posterior to canthals is slightly smaller than first canthal; row of slightly enlarged scales along anterior aspect of dorsolateral edge of orbit; circumnasal scale separated from rostral by one scale; interparietal length-SVL ratio 0.021; seven supralabials to center of eye; seven postmentals; six postros-

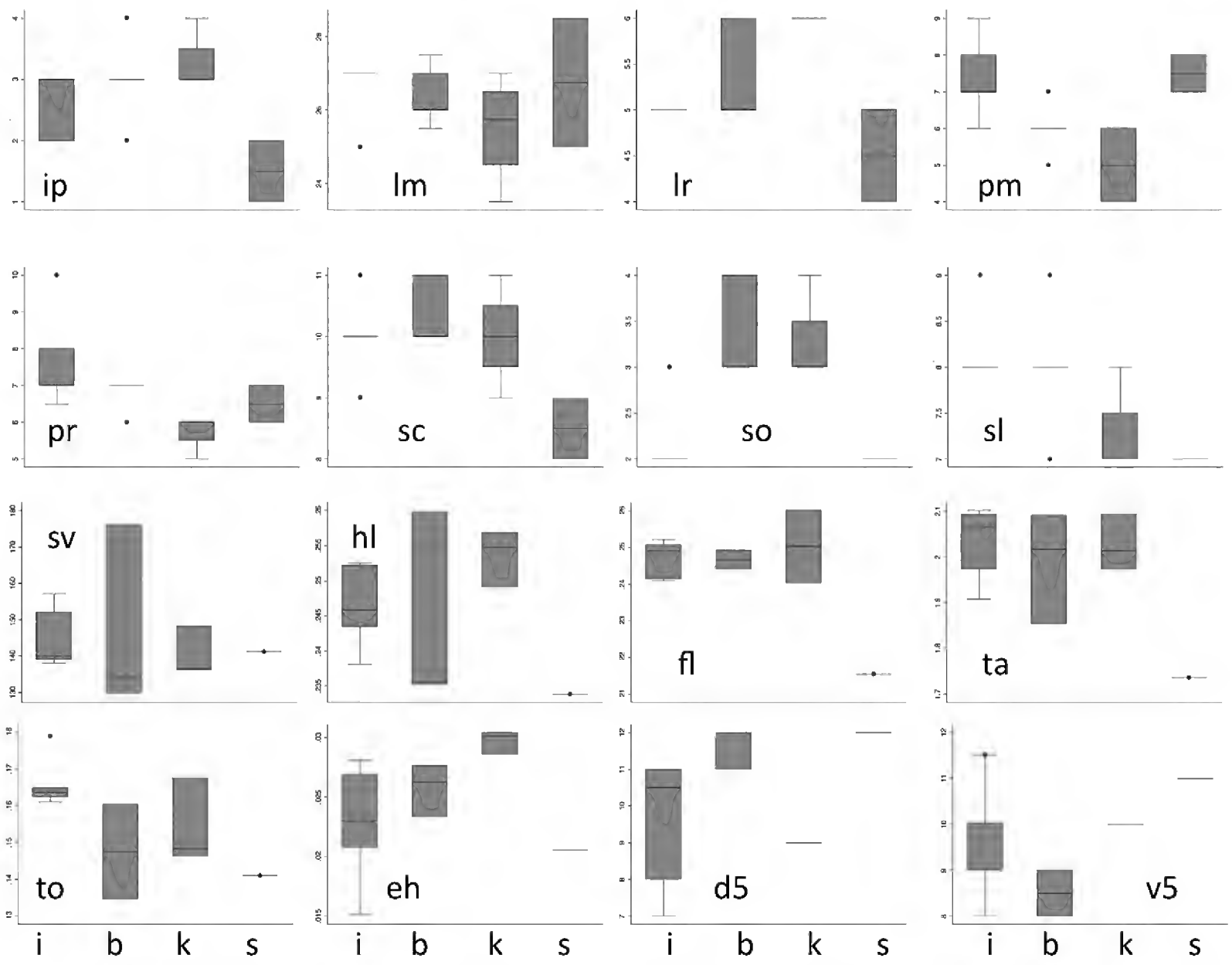

Fig. 9. Box plots showing variation between *Anolis insignis* (i), *A. brooksi* (b), *A. kathydayae* (k), and *A. savagei* (s). Traits are number of scales between interparietal and supraorbital semicircles (ip), number of expanded lamellae on fourth toe (lm), number of loreal rows (lr), number of postmental scales (pm), number of postrostral scales (pr), number of scales across the snout between the second canthals (sc), number of scales between the supraorbital semicircles (so), number of supralabial scales from rostral to center of eye (sl), snout to vent length (sv), head length relative to sv (hl), femoral length relative to sv (fl), tail length relative to sv (ta), toe length relative to sv (to), ear height relative to sv (eh), number of longitudinal dorsal scales in 5% of sv (d5), number of longitudinal ventral scales in 5% of sv (v5).

trals; some enlarged scales present in supraocular disc, decreasing gradually in size; mental partially divided posteriorly, with posterior border in concave arc; lateral edges of rostral extend farther posteriorly than mental; two enlarged smooth sublabials; more posterior lateral throat scales are keeled.

Dewlap reaches well posterior to axillae in males and females; dewlap scales in rows of multiple scales in both sexes; pair of distinct, abruptly enlarged postcloacal scales present; dorsal scales smooth, with no enlarged middorsal rows, 12 longitudinal rows in 5% of SVL; nuchal crest present with slightly enlarged middorsal scales; ventral scales in transverse rows, smooth, 11 scales in 5% of SVL; supradigitals multicarinate; toepads expanded; 28–29 lamellae under third and fourth phalanges of fourth toe; tail with a double row of middorsal scales; thigh scales smooth dorsally and ventrally, mostly smooth anteriorly with a few weakly unicarinate scales.

Color pattern in life

Color patterns of a male (MSB 96616) and female (UCR 20635) specimen were very similar. Dorsal color was generally brown, with alternating tan and dark brown irregular bands, the dark bands with some lighter blotching within them. Photographic evidence (R. Stanley, I. Latella; pers. comms.) indicates some individuals possess green and pale peach-orange dorsally in addition to brown. The dewlap in both sexes was pale pink with black horizontal streaks. No shoulder blotch was observed, but a prominent postorbital blotch was present in all adult specimens examined ($n = 5$).

Distribution and habitat

We found *Anolis savagei* at night sleeping 5–6 m up on narrow tree branches along trails in the closed canopy secondary forest of Las Cruces Biological Station. More work is needed on the ecology of this species. Specimens examined for this paper are from the Cordillera de Talamanca in southwestern Costa Rica at 1,127 m. Two individuals photographed from the western edge of Chirripó National Park at 1,590 m (R. Stanley, pers. comm.) apparently are *A. savagei* based on the presence of a prominent postorbital blotch in each, and the darkly streaked dewlap of the individual for which the dewlap is partially visible. We have not examined the *A. insignis*-like specimens reported from near sea-level by Savage and Talbot (1978; Ballena, BM 1909.7.10.20; Rincón de Osa, UCR 4387), but these are likely to be *A. savagei* based on

those authors' emphasis of a postorbital blotch in these specimens. Given these localities, *A. savagei* occurs on the Pacific slope of the Cordillera de Talamanca from sea level to at least 1,590 m, from Chirripó National Park south to Las Cruces (Fig. 8).

Anolis kathydayae, new species

(Figs. 2, 5, 6)

urn:lsid:zoobank.org:act:31E4F176-EA11-4172-A0E1-A9DE3AE65287

Holotype

MSB 96614 adult male from Panama, Chiriquí, trail from paved road near Chiriquí/Bocas del Toro province boundary at Fortuna pass; 8.78533, -82.21434, 1,178 m; collected by Steven Poe and Julian Davis on 13 March 2013.

Paratypes

MVUP 2128, juvenile from Panama, Bocas del Toro, side of Fortuna pass road, just north of Chiriquí/Bocas del Toro boundary; 8.78008, -82.20584, 1,038 m; collected by Steven Poe and Julian Davis on 13 March 2013. MSB 96612, same locality as holotype, collected by Steven Poe and Caleb Hickman, December 2003. MSB 79921, MSB 96613, same locality as holotype, collected by Steven Poe, Erik Hulebak, and Heather MacInnes on 28 July 2005.

Diagnosis

Anolis insignis, *A. brooksi*, *A. savagei*, and *A. kathydayae* are the only Central American *Anolis* to combine large size (> 120.0 mm SVL), smooth scales on the upper thigh, and short limbs (Savage and Talbot 1978). *Anolis kathydayae* is distinguished from these species by male dewlap color pattern (white with light green or dull blue tint in male *A. kathydayae*; orange-red in male *A. insignis*; pale pink with dark streaks in *A. savagei*; peach-tan in *A. brooksi*; Figs. 1, 2). It is further distinguished from *A. savagei* and *A. brooksi* by female dewlap color pattern (solid white with greenish tint in *A. kathydayae*; white or brown with dark streaks in *A. brooksi*; pale pink with dark streaks in *A. savagei*; unknown in *A. insignis*). At least in our samples, *A. kathydayae* is further distinguished from *A. insignis* by several scale characters (Table 1; e.g., fewer postmentals, 4–5 versus 6–9 in *A. insignis*). Additionally, the two male *A. kathydayae* we have examined display obscure, weakly enlarged postcloacal scales, whereas all male individuals of the other *insignis*-like anoles we have examined display large, distinct postcloacal scales.

Etymology

The name is a matronym to honor Kathy Day and the Miller Institute for Basic Research in Science. Kathy has contributed greatly to the professional and personal development of scientists and the advancement of basic science through her position running the Miller Institute.

Description of holotype

Snout-vent length 148.0 mm; head length-SVL ratio 0.26, head width-SVL ratio 0.15; ear height-SVL ratio 0.030; femoral length-SVL ratio 0.26; tail length-SVL ratio 2.0. Dorsal head scales mostly smooth, some with weak keels or wrinkling reflecting underlying bone or ossification; frontal depression present, dorsum with weak parallel rows evident anteriorly; rostral overlaps mental anteriorly; 10 scales across snout between second canthals; four scales between supraorbital semicircles; subocculars in contact with supralabials; zero elongate superciliary scales; first scale posterior to canthals is smaller than first canthal; six loreal rows; circumnasal scale separated from rostral by one scale; interparietal length-SVL ratio 0.018; seven supralabials to center of eye; six postmentals; six postrostrals; some enlarged scales present in supraocular disc, decreasing gradually in size, bordered medially by a partial row of small scales; mental partially divided posteriorly, extending posterolaterally approximately even with rostral, with posterior border in concave arc; one-two enlarged keeled sublabials.

Dewlap reaches well posterior to axillae in males and females; dewlap scales in rows of multiple scales in both sexes; no axillary pocket; postcloacal scales slightly enlarged; dorsal scales smooth, pair of middorsal scale rows slightly raised, nine longitudinal rows in 5% of SVL; nuchal crest present with pair of slightly enlarged triangular middorsal scale rows; ventral scales in transverse rows, smooth, 10 scales in 5% of SVL; supradigitals multicarinate; toepads expanded, 27 lamellae under third and fourth phalanges of fourth toe; tail with a double row of middorsal scales; thigh scales smooth to weakly keeled dorsally and ventrally, unicarinate anteriorly, multicarinate at knee.

Color pattern in life

An adult male (MSB 96614) had a tan body with discrete dark green broad bands speckled with light tan. The anterior body to posterior head had a bluish-green wash. Dorsal head scales were greenish-tan, outlined with darker brown. A very faint blotch was present above the shoulder. The iris was brown and the tongue was dark yellow. The limbs and digits were greenish-tan, with darker green bands. The tail was banded with sharply alternating black and tan bands. The dewlap was white, with a yellowish-green tint. Another adult male (MSB 96613) was patterned similarly but mostly lacked green—the anterior bluish-green wash was absent, and the bands were dark brown to black with no greenish tint. The dewlap of this individual was white, with faint blueish tint. One adult female (MSB 79921) appeared dark greenish with diffuse banding of white, darker green, and brown. The dewlap appeared very pale yellow-green. A juvenile female (SVL 87.0 mm; MSB 96612) appeared nearly completely pale green, with faint white lateral bands and

some darker green reticulations on the body and darker green bands on the limbs and digits, and white blotches dorsally on the head. This individual had a pale greenish-yellow dewlap with some dark green reticulations. A near-hatchling (MSB 96615) had a cream dewlap with prominent black streaks.

Distribution and habitat

We found adults of *Anolis kathydayae* sleeping horizontally on narrow branches along a trail in secondary forest three to five meters above the ground, and juveniles at roadside habitat four to five meters above the ground on twigs. Elevational range of these two sites is 1,038–1,178 m. Currently known distribution for *A. kathydayae* is the Fortuna pass area of Panama.

Discussion

The four *insignis*-like *Anolis* species discussed here are distinct in male dewlap color (Figs. 1, 2), which usually varies little within species of *Anolis*, and in additional morphological traits (Diagnoses; Table 1; Fig. 9). Below we discuss the status of each species relative to previous discussions on these forms and our own views of the distinctiveness and importance of diagnostic traits for these species, especially in light of our small sample sizes. We also discuss some limited molecular data bearing on these forms.

Savage and Talbot (1978) originally drew attention to differences between Northern Costa Rican (i.e., *Anolis insignis*), southern Costa Rican (i.e., *A. savagei*), and Panamanian (i.e., *A. brooksi*, *A. kathydayae*) "*A. insignis*." The postocular blotch of southern Costa Rican forms discussed by these authors appears to be an autapomorphic diagnostic trait for *A. savagei*. Including photos, preserved specimens, and reports from Savage and Talbot (1978), we are aware of eight specimens that are assignable to *A. savagei* based on male dewlap color of the population and locality. All eight of these specimens possess a postocular blotch, and all *A. insignis*, *A. brooksi*, and *A. kathydayae* examined by us (including photos, $n = 18$) lack a postocular blotch. Additionally, *A. savagei* is quite distinct in overall morphology (Table 1; Diagnoses; Fig. 9).

Anolis kathydayae is striking in its possession of pale, patternless dewlaps in males and females (Fig. 2). Although a few species of *Anolis* display intraspecific variation in male dewlap color pattern, such variation nearly always occurs within populations (e.g., *A. gemmosus* around Mindo, Ecuador; *A. valencienni* in northern Jamaica) or at hybrid zones (e.g., *distichus*-group forms; Glor and LaPort 2012). Thus we note the relative invariance of the distinctive male dewlap of *A. brooksi* across El Copé in Cocle (Fig. 2), Santa Fe in Veraguas (Fig. 7), Cerro Azul in Panama (Fig. 7), and possibly Isla Escudo de Verguas in Bocas del Toro (pers. obs.; see above) as evidence for the species status of this form relative to the other forms discussed here. We note the constancy of the distinctive streaked dewlap of *A. savagei* between Las Cruces and Chirripó (a distance of ~100 km), and the presence of an orange-red male dewlap of *A. insignis* over at least three localities in northern Costa Rica (Poco Sol, La Fortuna, Monteverde; photographic evidence). We know of no intermediate forms between these dewlap types, although some minor variation occurs within each of them. Thus we view the presence of the unusual male and female dewlaps of *A. kathydayae* as strong evidence for the species status of this form, in addition to the molecular evidence presented below and the external morphological patterns shown in Table 1 and Fig. 9.

We observed three of the four species of *insignis*-like anoles to differ consistently in female dewlap color (Fig. 2). Female *Anolis brooksi* have a white or brown dewlap with black streaks, female *A. savagei* have a pale pink dewlap with dark streaks, and female *A. kathydayae* have a pale, patternless dewlap (we have not seen a confirmed female dewlap of true *A. insignis*). We note that there is considerable ontogenetic variation in this trait, with all examined juvenile females in life (*A. kathydayae*, *A. brooksi*) possessing some dark streaking on the dewlap. Our observations of adult female dewlap color pattern suggest some taxonomic utility to this character in this case, but these differences may not be evident in larger sample sizes.

The Northern Costa Rican form (i.e., *Anolis insignis*) and the widespread Panama form (i.e., *A. brooksi*) share similar dorsal color patterns and their male dewlaps are most similar among the species discussed here (Figs. 1, 2). There remains much work to be done on the systematics of these forms. The geographic patterns among the *insignis*-like *Anolis*, including two similar geographically intervening species (i.e., *A. savagei*, *A. kathydayae*; Fig. 8), suggests that conspecificity of *A. brooksi* and *A. insignis* is unlikely. Still, this is a hypothesis that begs continued investigation, as is the potential presence of multiple species within *A. insignis* and *A. brooksi*. In particular, we have little confidence that the populations that we are calling *A. brooksi* are actually conspecific with topotypical *A. brooksi*, for which we have examined only a single preserved juvenile specimen (i.e., the holotype). We elect to use this name because juveniles of the tan-dewlap form (i.e., *A. brooksi* as we are recognizing it) are indistinguishable from the holotype of *A. brooksi*, and the range of the tan dewlap form approaches the *A. brooksi* type locality to the east. To give the tan-dewlap form a new name rather than assume its conspecificity with *A. brooksi* seems unconservative under these circumstances.

The low sample sizes of our analyses (Table 1; supplemented by photographic evidence and observations in Savage and Talbot [1978] and Lotzkat et al. [2013]) are unfortunate but currently unavoidable. The *insignis*-like *Anolis* apparently are difficult to find, or possibly rare. Lotzkat et al. (2013) included just two collected individ-

uals of *insignis*-like anoles in their summary of the giant anoles of Panama. Savage and Talbot (1978) studied all specimens of *insignis*-like anoles collected before 1978, a total of 24 individuals. Vertnet lists just 28 records for *A. insignis* as of 08 August 2016, after decades of intensive herpetological field work in Costa Rica and Panama since Savage and Talbot (1978). Our new sample of eleven collected specimens, plus additional photographic vouchers, warrants a new treatment of these forms and supports recognition of multiple species. However, we recognize that the strength of our inferences is tempered by our necessarily limited sampling. We have little doubt that the taxonomic picture we have painted for these forms, while pragmatic and warranted given the evidence in front of us, is incomplete.

Some DNA sequence data has been generated for *Anolis brooksi* and *A. kathydayae* under the name *A. insignis*, but no molecular data exists for *A. savagei* and true *A. insignis*. Castañeda and de Queiroz (2011) included data from COI, ND2, and RAG1 genes for an "*A. insignis*" sample from Fortuna Reserve, i.e., near the type locality of *A. kathydayae*. Alfoldi et al. (2011) included data for several genes for a sample of *A.* "*insignis*" from Cerro Azul, Panama Province (POE 2154 in their appendix; now MVUP 2007). This individual clearly is assignable to *A. brooksi* (Fig. 7). Lotzkat et al. (2013) collected 16S data for an adult and juvenile female specimen of "*A. insignis*" from Santa Fé, Veraguas, and Willie Mazu, Comarca Ngöbe-Buglé in Panama, respectively. Accurate identification of these specimens is not straightforward because our diagnoses are based mainly on adult male specimens and the species in question generally overlap in scalation (Table 1). However, the adult female specimen of Lotzkat et al. (2013), from Santa Fé, is referable to *A. brooksi* based on female dewlap color pattern (Lotzkat et al. 2013: Fig. 14C) and locality; a subadult male photographed from Santa Fé (Fig. 7) clearly is *A. brooksi*. The juvenile specimen (SMF 91477) may be *A. kathydayae* or *A. brooksi*. The locality of this specimen is proximal to the type and other known locality of *A. kathydayae* but at a lower elevation on the Caribbean slope. This proximity to the *A. kathydayae* type locality suggests *A. kathydayae* as the most likely identification for this population, but reported 16S distances suggest this sample represents *A. brooksi*. The uncorrected 16S distance between the Lotzkat et al. (2013) samples is just 0.004—a 16S distance corroborated by comparison of the Willie Mazu sequence with our Santa Fé sample (MVUP 2007). Perhaps this specimen is *A. kathydayae* and 16S is evolving slowly in one or both of *A. kathydayae* and *A. brooksi*, or perhaps the specimen is *A. brooksi* and this species approaches *A. kathydayae* on the Caribbean slope.

An alternative interpretation of the 16S result is conspecificity of the Fortuna and Santa Fé populations (i.e., of *Anolis brooksi* and *A. kathydayae* as we have recognized them here), with the differences between these populations noted herein attributed to intraspecific variation. This interpretation seems unlikely given the consistent morphological differences between these forms (Fig. 2; Table 1; Fig. 9) and new information on mitochondrial DNA distances for these populations. We sequenced the mitochondrial ND2 gene of the Santa Fé tissue (data included here in the phylogenetic analysis) as part of a larger project (Poe et al. 2017) and found an uncorrected ("p") distance of 12.5% between the Castañeda et al. (2011) "*A. insignis*" sample (i.e., *A. kathydayae*) and the Santa Fé sample (i.e., *A. brooksi*). This distance is similar to pairwise species distances among many distinctive species of *Anolis* (e.g., the *A. microtus-A. brooksi* [Santa Fé] ND2 distance is 9.5%). Thus, information from the ND2 gene corroborates our morphological inference of separate species status for Fortuna (*A. kathydayae*) and eastern (*A. brooksi*) populations of anoles similar to *A. insignis*.

The phylogenetic analysis was unable to robustly resolve the relationships of the new forms (Fig. 4). The well-supported clades in the estimated tree—i.e., the ingroup and the sister relationship of *Anolis microtus* and *A. ginaelisae*—were well-established previous to this work (Savage and Talbot 1978; Castañeda and de Queiroz 2011; Lotzkat et al. 2013; Poe et al. 2015). The poor support for the interrelationships of the four species discussed here indicates that external morphological data alone is inadequate to resolve them. Clearly, additional phylogenetic work using DNA sequences is needed on the *insignis*-like *Anolis*. Fresh sampling of known coastal versions of these species in Caribbean Panama and Pacific Costa Rica (Fig. 8; see localities in Savage and Talbot [1978]) and incorporation of material from the type localities of *A. insignis*, *A. savagei* and *A. brooksi* would be especially informative, for questions of species boundaries as well as phylogeny.

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Appendix 1

Morphological characters for phylogenetic analysis.

1. Maximum snout to vent length (SVL; mm; ordered). 0: < 120; 1: 120–129; 2: 130–139; 3: 140–149; 4: 150–159 5: >159.

2. Femoral length/SVL (ordered). 0: < 0.230; 1: 0.230–0.239; 2: 0.230–0.239; 3: 0.240–0.249; 4: 0.25–0.259; 5: >0.259.

3. Ear height/SVL (ordered). 0: < .017; 1: 0.17–0.019; 2:0.020–0.022; 3: 0.023–0.025; 4: 0.026–0.028; 5: >0.28.

4. Toe length/SVL (ordered). 0: < 0.16; 1: 0.16; 2:0.17; 3: 0.18; 4: 0.19; 5: >0.19.

5. Tail length/SVL (ordered). 0: < 1.75; 1: 1.75–1.84; 2: 1.85–1.94; 3: 1.95–2.04; 4: 2.05–2.14; 5: >2.14.

6. Mean number of longitudinal ventral scales in 5% of SVL (ordered). 0: < 8; 1: 8–8.4; 2: 8.5–8.9; 3: 9–9.4; 4: 9.5–9.9; 5: >9.9.

7. Mean number of longitudinal dorsal scales in 5% of SVL (ordered). 0: < 8.5; 1: 8.5–8.9; 2: 9–9.4; 3: 9.5–9.9; 4: 10–10.4; 5: >10.5.

8. Mean number of expanded lamellae on toe IV (ordered). 0: < 23; 1: 23; 2: 24; 3: 25; 4: 26; 5: >26.

9. Mean number of scales across the snout at the second canthals (ordered). 0: < 7; 1: 7-7.9; 2: 8–8.9; 3: 9–9.9; 4: 10–10.9; 5:>11.

10. Mean number of scales between supraorbital semicircles (ordered). 0: 0: < 2; 1: 2; 2: 2.5; 3:3; 4: 3.5; 5:>3.5.

11. Elongate superciliary scale (longer than first canthal; frequency-coded). 0: absent; 5: present.

12. Mental (frequency coded). 0: extends along mouth posteriorly past rostral; 5: rostral extends posteriorly past mental.

13. Mean number of postmental scales (ordered). 0: < 6; 1: 6–6.4; 2: 6.5-6.9; 3: 7–7.4; 4: 7.5-7.9; 5: >7.9.

14. Number of postxiphisternal incriptional ribs (Etheridge 1959; Savage and Talbot 1978; frequency coded). 0:4; 5:5.

15. Number of supralabial scales from rostral to center of eye (ordered). 0: < 6.5; 1: 6.5-6.9; 2: 7.0-7.4; 3: 7.5-7.9; 4: 8.0-8.4; 5: >8.4.

16. Scales on upper surface of thigh (Savage and Talbot 1978; frequency coded). 0: smooth; 5: keeled.

17. Scales in supraocular disc (Savage and Talbot 1978; ordered). 0: small, approximately equal in size; 5: mix of large and granular scales.

18. Male dewlap color (unordered). 0: pink; 1: white; 2: orange-red; 3: tan-peach; 4: pale pink with black streaks; 5: yellow.

Appendix 2

Coding for morphological characters in phylogenetic analysis.

A. fraseri	0	1	3	1	5	1	5	0	2	1	(23)	0	1	0	5	5	0	5
A. frenatus	3	5	4	5	4	5	4	3	5	5	5	0	5	0	5	5	0	1
A. ginaelisae	0	4	0	3	5	2	0	0	1	0	0	0	0	5	3	5	5	0
A. microtus	1	2	0	3	4	0	0	0	0	1	0	0	1	5	2	5	5	0
A. insignis	4	4	3	2	3	4	3	5	4	1	0	4	3	5	4	0	5	2
A. brooksi	5	4	4	0	3	2	5	4	4	3	0	0	1	5	4	0	5	3
A. kathydayae	3	4	5	0	3	5	2	4	4	3	0	4	0	5	2	0	5	1
A. savagei	3	0	2	0	0	5	5	5	2	1	0	5	4	5	2	0	5	4

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Stakeholder contributions to conservation of threatened Northern Pine Snakes (*Pituophis melanoleucus*, Daudin, 1803) in the New Jersey Pine Barrens as a case study

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***Abstract.*—The successful management and protection of endangered or threatened species generally falls to state agencies. This paper suggests that while governmental agencies provide the legal, regulatory, and management framework for snake conservation, it is often the universities, conservation organizations, consultants, and concerned citizens that conduct the research needed for conservation efforts. Identification of all the relevant stakeholders and their contributions is important for determining how to manage the threats and enhance population viability. Managing the efforts of volunteers is hampered by the need to protect the locations of sensitive nesting and hibernation habitat, while encouraging protection of the species overall. In this paper we provide a template of the stakeholder categories that are often involved in research, management, and conservation, and describe the types of agencies, organizations and people within each category and their major contributions, using research with Pine Snakes (*Pituophis melanoleucus*). This suite of stakeholders has been successfully involved with Pine Snake research for over 30 years, and helped with examining key environmental and habitat needs. The contributions are synergistic and additive, lending continuity of stakeholder involvement. We also suggest several stakeholder involvement actions that can be useful to a range of conservationists.**

Keywords. Environmental management, management framework, public participation, sensitive species, reptiles

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Introduction

Initially, decision-making and managing environmental resources was a top-down approach, where the involvement of the public in research and conservation was largely one way, with governmental agencies providing information to the public. This evolved into two-way communication where agencies also asked the public for their input, perceptions, and concerns. The importance of stakeholders and communities in environmental management was initially acknowledged in the Environmental Protection Agency's risk assessment paradigm, which included the public in the problem formulation phase (USEPA 1992, 1998). Several subsequent authors recognized the importance of a multi-stakeholder framework for environmental management, where a range of stakeholders was involved in goal-setting for a project (Pittinger et al. 1998). The Presidential/Congressional Committee on Risk Assessment and Risk Management (PCCRARM 1997) acknowledged that the National Research Council's (NRC 1983, 1996) risk assessment paradigm required the addition of stakeholders and risk management to the process. Public participation or involvement is usually monitored as the success of the process, or the success of the project (Chess and Purcell 1999), but not the success of stakeholder inclusion.

The realization of the importance of stakeholders in decision-making was empowering, and has led directly to the involvement of stakeholders in every phase of monitoring, assessment, research, and conservation (Bon-

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Fig. 1. Northern Pine Snake (*Pituophis melanoleucus*) hissing when first encountered in the New Jersey Pine Barrens.

ney et al. 2009; Glowinski and Moore 2014). Partly the stakeholder participation derived from analysis of ecosystem services and governance (Paavola and Hubacek 2013). Three major advances followed: 1) stakeholder was defined as all interested and affected parties, including governmental agencies, non-governmental organizations, the private sector, and the general public, 2) stakeholders could identify environmental issues and formulate the questions requiring answers, and 3) a wide range of stakeholders could be involved in all phases of designing and implementing an environmental management project. Although the last is an ideal approach, it is seldom achieved in practice. Stakeholders may be particularly important to predicting or deducing unintended consequences of management. Yet, with decreasing federal, state, and local personnel, and decreasing and limited funding, involving a wide range of stakeholders in projects to help conduct studies and participate in environmental management and conservation is an ideal method of accomplishing more with less, while gaining public support. Citizen science projects, and community participatory research, are becoming more common and more powerful (Bonney et al. 2009; Dickinson et al. 2010). Citizen science is a method of integrating public outreach and scientific data collection locally and regionally (Cooper et al. 2007). An important aspect of citizen science is to gather natural history information that might otherwise go unnoticed (Dickinson et al. 2010). Stakeholder involvement, whether identified as citizen science or participatory research offers opportunities (Conrad and Hilchey 2011), particularly for conducting long-term studies and monitoring for sustained conservation efforts (see Lawrence 2006).

In this paper we describe the risks faced by Pine Snakes (*Pituophis melanoleucus*) as a case study to identify the types of stakeholders that can be involved in snake research and conservation (Fig. 1). We also give examples of each type, and provide descriptions of the different types of contributions that stakeholders can make that lead to understanding the biology and conservation needs of snakes. Assessing stakeholder participation can lead to increases in the wise use of professionals and volunteers, but can also provide examples of opportunities to engage people and use personnel, and provide models of participation for others engaged in management of natural resources. This is a recently developed, often overlooked approach that can increase the personnel and provide logistic support needed to conduct long-term research. The threats in urban areas are partly offset by the potential for many volunteers. This approach has the added advantage of increasing public awareness, knowledge, and appreciation for snakes in general. The popular jargon for volunteers is citizen scientists (Cooper et al. 2007; Dickinson et al. 2010), but using a range of stakeholders involves more than just volunteers. Including stakeholders in management is particularly important, given the global decline of reptiles in general (Gibbons et al. 2000).

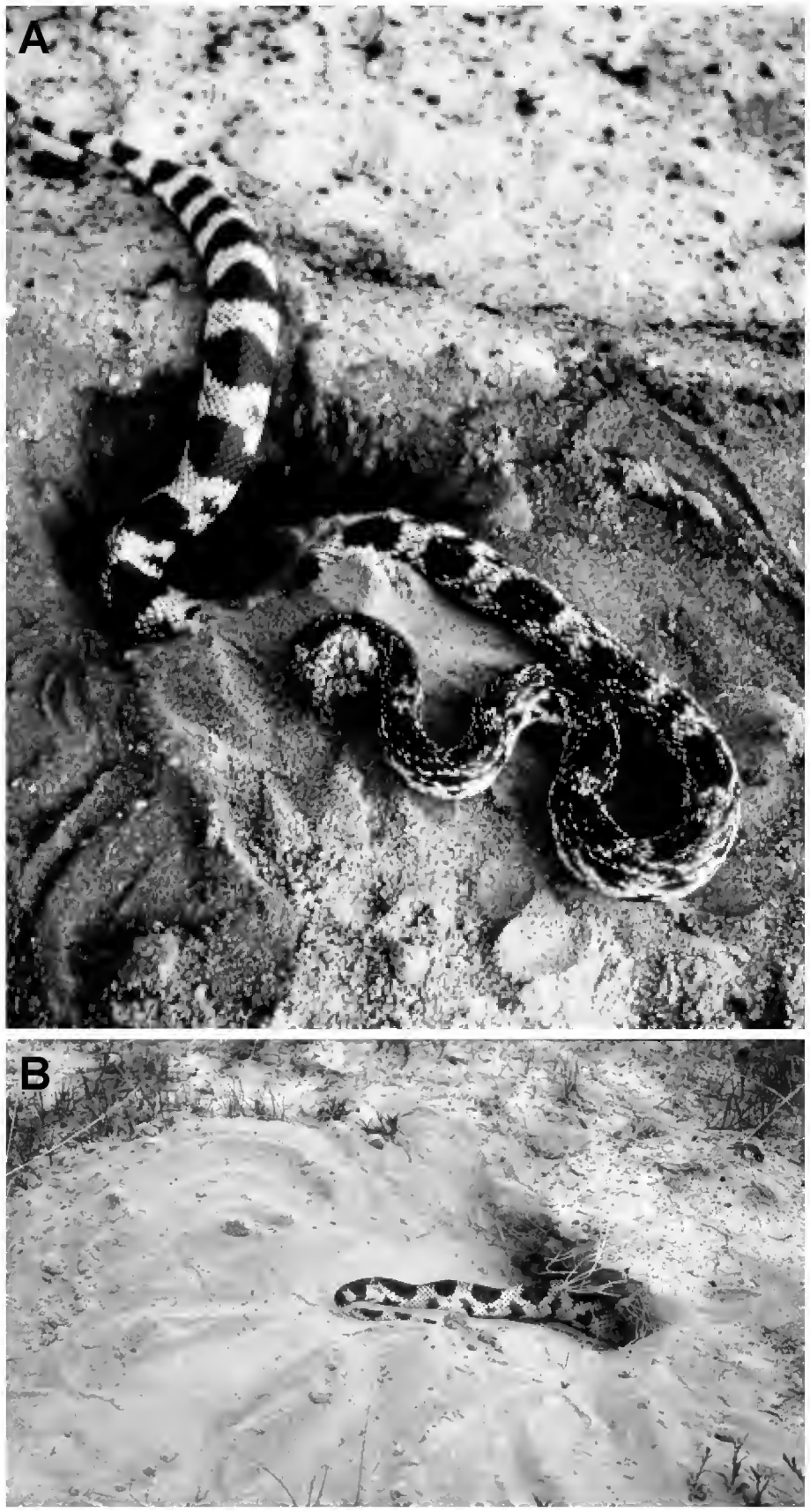

Fig. 2. Female Northern Pine Snakes dig their own nests in the New Jersey Pine Barrens, although in the southern part of their range they do not do so. They bend their neck such that the head forms a scoop capable of bringing sand out the entrance (**Fig 2a**). While digging their body is hidden below ground, and the dump pile of sand is visible (and serves to attract poachers; **Fig 2b**).

Background on Pine Snakes: Northern Pine Snakes are large constrictors that reach the northern limit of their range in the New Jersey Pine Barrens. They are among the top-level predators in the region and can grow to almost two meters long (Conant and Collins et al. 1998; Powell et al. 2016; Burger and Zappalorti, unpub. data). This species is declining in many parts of its range, and is not common anywhere. The declines of the species to the south, and its threatened status in New Jersey, make it imperative to understand the factors impacting population levels. The New Jersey population of Northern Pine Snakes is isolated from other populations living to the south by several hundred km (Burger and Zappalorti 2011a, 2016; Powell et al. 2016).

Fig. 3. Typical nesting area of Northern Pine Snakes in New Jersey. They require relatively open areas where there is complete sun penetration to the ground to provide sufficient warmth to the incubating eggs (Burger 1989a, 1991a; Burger and Zappalorti 2011a).

Fig. 4. Female Pine Snakes sometimes remain in their nests for several days after egg-laying is complete, perhaps protecting their clutch from being disrupted by other females that lay in the same nest.

Pine Snakes in the New Jersey Pine Barrens are the only North American snake that excavates their own nest in open-canopy sandy areas, and show high fidelity to these exact nest sites (Burger and Zappalorti 1991, Fig. 2). Open sandy areas with appropriate ground vegetation to provide structure to support excavation, while maintaining sun penetration to the ground, are rare in the Pine Barrens. Usually several females nest in the same open clearing (Fig. 3), and sometimes several females lay eggs in the same nest (Burger and Zappalorti 1991, 1992). The nest tunnel can be more than two meters long. Clutches can be distinguished because females exude a substance that binds the eggs together. Excavation of nests can take several days, and digging females usually rest during the hottest part of the day in the shade of pine trees. Once part of the tunnel is excavated, females sometimes remain in the tunnel during the heat of the day, and continue to do so for a few days after a clutch is laid (Fig. 4). Nesting females and their nests are vulnerable to off-road vehicles (ORVs), poachers, and predators, as are hatchlings (Burger 2006, 2007, Burger et al. 1992, 2007;

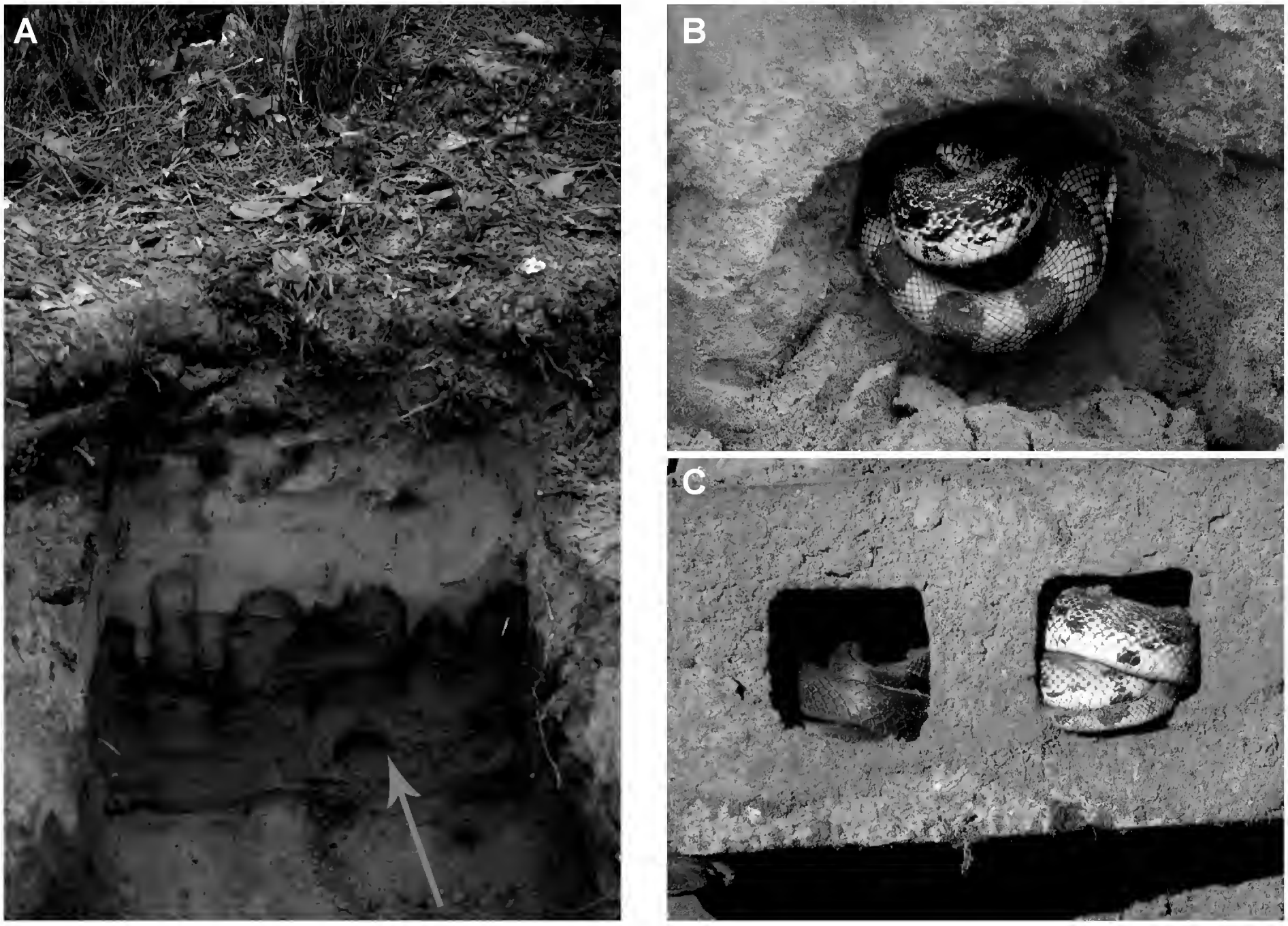

Fig. 5. Pine Snakes hibernate in communal hibernacula that can contain up to 30 or more Pine Snakes (Burger et al. 1988; Burger and Zappalorti 2011a, b, 2015, 2016). **Fig. 5a** shows the depth hibernation chambers are below ground, a snake in a natural chamber (**Fig 5b**) and in cement blocks from an old septic chamber (**Fig. 5c**, Pine Snake on right, Black Racer on left).

Burger and Zappalorti 2016). Northern Pine Snakes from the New Jersey Pine Barrens are highly prized by collectors because of their vibrant black and white pattern.

Hatchlings emerge in the late summer or early fall, and find their way to hibernacula by following adult scent trails (Burger 1989a, 1990), or they hibernate in old stump holes or other places. Adults have relatively large territories, and radio-tracked snakes can be found as far as 3–4 km away from hibernation and nesting areas (Burger and Zappalorti 2011a, Zappalorti et al. 2014, 2015).

Snakes spend the winter in communal hibernacula that they modify from old mammal burrows and old stumps, digging long tunnels out into virgin sand, and overwintering in chambers (Burger et al. 1988; Burger and Zappalorti 2011a, 2015, 2016). The snakes usually hibernate a meter or more below the ground in chambers the size of their coiled body (Fig. 5). Traditional hibernacula are used for many years, and several we study have been active for 30 + years. If a hibernaculum is entered by mammalian predators, it may be abandoned for several years, but snakes eventually return to use it (Burger and Zappalorti 2011a). Both sexes show philopatry to hibernation sites, but females are more philopatric than males (Burger and Zappalorti 2015). Once we have dug up a hibernacula, we rebuilt it with an appropriate chamber and entranceway made of cement blocks that prevent mammalian predators from entering. Our marking and recapture methods have not adversely affected the behavior or survival of the snakes (Burger and Zappalorti 2011b).

Northern Pine Snakes are vulnerable to the usual threats of insufficient food supplies, predators, inclement weather, and finding hibernation sites (this is especially true for hatchlings), but they also face human disturbance, wanton killing, mortality on roads, and poaching. They are vulnerable due to habitat loss and fragmentation, and human activities that lead to local extirpations (Golden et al. 2009; Burger and Zappalorti 2011a; 2016). It is for this reason that the involvement of a full range of stakeholders (including the public) is necessary and important to the conservation of this large snake. Involvement of stakeholders is an important aspect of the Pinelands National Reserve management (New Jersey Pinelands Commission 2009).

Materials and Methods

The objectives of this series of studies of Pine Snakes, which has spanned over 40 years, are to 1) examine the

breeding and hibernation biology of Pine Snakes, 2) understand the threats faced by Pine Snakes, and gather information helping to preserve them, 3) understand the possible role of contaminants, 4) conserve Pine Snake populations in their preferred habitats, and 5) educate the public about the importance and role of Pine Snakes in the Pine Barrens ecosystem. Over the last 30 years as it became clear that people, organizations and agencies wanted to contribute, and to take part in a research and conservation efforts to conserve Pine Snakes. Our intent is to describe the various contributions of different organizations and people to serve as an example for other short or long-term studies with reptiles, whether threatened or not. All procedures were completed under appropriate state permits and a Rutgers University protocol approval (E86-017).

Results

Types of stakeholders: Understanding the biology of species, and collecting data for management and conservation traditionally fell to governmental agencies and universities. However, many different categories of stakeholders now participate and fund species conservation and management. Table 1 lists the categories that are relevant for Northern Pine Snakes, and that have participated in Pine Snake research and conservation activities to a greater or lesser degree. A general description of each stakeholder type follows, and may be useful for other species of conservation concern (Table 1). This represents a suite of stakeholders that may be involved in many different types of environmental studies.

Stakeholder contributions to Pine Snakes conservation: Within each stakeholder type there are different organizations, groups, and individuals that contribute to research and conservation of Pine Snakes in the New Jersey Pine Barrens. Some stakeholder groups contribute positively, without any negative effects, while others can have both positive and negative effects on Pine Snakes (usually not the same people). Tables 2 and 3 list the threat types, and the roles of stakeholders' in conservation and research in the New Jersey Pine Barrens. The references in Table 3 generally relate to Northern Pine Snakes in the New Jersey Pine Barrens (or from other regions), and not to other congeners. Much of the information available for Pine Snake life history and behavior comes from either university studies, or those funded by state agencies or industry, or a combination thereof, with the help of volunteers (Fig. 6).

Discussion

Stakeholder involvement: Federal and state agencies (resource and regulatory) are usually thought of as determining the status and trends of animals, protecting and conserving them, regulating or permitting their use, and conducting research that leads to conservation and management. With limited and sometimes declining resources, agencies must set priorities, and different agencies may have conflicting priorities (i.e., promoting multiple use vs protecting resources). While State involvement has been valuable for Pine Snake conservation, there are other groups that play critical roles in research and conservation. These roles are essential

Table 1. Types of stakeholders that can participate in research and conservation. Not all species, populations, or communities will have this full range of stakeholders.

Type	Definition
Independent Scientist (university, museum, other)	Scientist engaged in designing and implementing research projects, leading to public talks, publication and dissemination of results, and in some cases, to regulations or adaptive management.
Natural Resource Agency	State, federal, or local agency responsible for managing a biological resource (a species, population, community, natural area, preserve, or ecosystem)
Management Agency	State, federal, or local agency responsible for managing a resource other than biological one (e.g., water authorities)
Regulatory Agency	State, federal, or local agency responsible for developing and enforcing regulations that pertain to a species, population, community, or ecosystem (e.g., park, refuge), as well as media resources (e.g., water).
Conservation Organization	Non-governmental agency (NGO) with a conservation mission to protect species, populations, communities, or ecosystems, including endangered and threatened species. Can be national, state, or local.
Other Non-governmental Agency	Any other NGO with a vested interest in the species, population, community, or ecosystem, either directly or indirectly.
Environmental Justice Community	Any identifiable environmental justice community that is interested or affected by the resource; usually involves low income or minority communities.
Public	The general public, not otherwise engaged in any of the above categories, that is interested and affected by the existence of a wildlife resource and the opportunity to experience it.
Consultant	Business specifically set up with expertise to address environmental questions posed by governments, industry, or developers.
Industry	Local or regional industry that overlaps in some way with a resource, through land, air, or water, or directly with a species or community.
Developer	Entity that develops or changes the local or regional land use, usually for residential or commercial activities.

Fig. 6. Volunteers of all ages are involved in our Pine Snake research, and the handling and measuring of snakes contributes to their education, and results in their providing information about conservation to their families, friends, classmates, and others. Following hibernation studies, the children (and adults) put the snakes back into their hibernation chambers.

because the NJDEP, Endangered and Nongame Species Program has insufficient resources to gather data on all the threatened and endangered species in the state. The trend of decreasing resources may continue.

Engaging the members of conservation organizations and the public in research activities has the added advantage in that they often become committed to continued work, to spreading conservation information, and to specifically protecting Pine Snakes (and other snakes). For many naturalists and conservationists, working with state and university scientists provides a unique and rare opportunity to work with endangered or threatened species, which is both rewarding and thrilling, while contributing to essential conservation knowledge. Allowing children, especially teenagers, to participate results in disseminating information and enthusiasm to their classmates and friends (Fig. 6). It also increases their awareness of the importance of Pine Snakes and preserving their environments.

The inclusion of stakeholders that participate in data collection can result in connecting people to information about the species around them (Lawrence 2006), as well as increasing and expanding scientific literacy (Bonney et al. 2009). These are valuable goals, particularly for snakes, which often are feared (and therefore killed or discouraged from urban areas). Partnerships among different agencies and conservation organizations can lead to both improved conservation of species, and to increased collaboration among entities that will benefit future conservation efforts (Bidwell and Ryan 2006). Stakeholder involvement can have the added benefit of demonstrating the adverse effects of some species (Young et al. 2013), such as raccoons, that have increased because of human provision of food in urban environments, especially on sensitive, threatened Pine Snakes. More case studies on stakeholder involvement in species conservation in urban areas could lead to some general principles of involvement. For example, people living along canals could monitor and track water snake numbers or their nest success, or people living near parks could track the number or habitat use of local snakes. Others in the public could record the location and date of turtle nests, of local species, or place protective cages over nests to prevent predation. In all cases, volunteers should coordinate with scientists and local agencies (Fig. 7).

Problems with involving stakeholders in conservation of a threatened species: There are several issues in involving many different stakeholders: 1) Protection of sensitive areas for Pine Snakes, 2) Protecting information about sensitive locations, 3) Conflicts among and within stakeholder groups, and 4) Securing help for field work when needed. In addition, illegal activities threaten the Pine Snake populations. Each will be discussed below.

The locations of sensitive areas for Pine Snakes need to be protected because they can be exposed to snake collectors that poach eggs, gravid females, and all Pine Snakes they encounter. With 6-digit GPS locations available on cell phones, this has become critical. Participants must be aware of the need to protect location data. In some years we have lost 40 % of our Pine Snake nests to poachers; the average was 29 %/year (Burger et al. 1992; Burger and Zappalorti 2011a). This is in addition to losses to natural predators such as foxes, raccoons, and skunks. It is imperative that everyone actively helping with Pine Snake work and conservation be aware of the potential, and avoid intentional or inadvertent disclosure of the location of nesting and hibernating snakes. This includes cautioning volunteers to avoid putting any information on social media that could indicate such locations, and warning them to turn off the GPS on their cameras and cell phones. People readily agree with this, but often are not aware of the problem. We are combating poaching by removing clutches before poachers have a chance to collect them. We hatch the eggs in the laboratory, and replace the hatchlings in their original nests after they

Table 2. Main threats faced by Pine Snakes in the New Jersey Pine Barrens and Opportunities for Stakeholder Involvement. These are not exhaustive, but provide examples of major threats or risks to the snakes.

Threat Type	Major Threat	Opportunity for Stakeholder Involvement
Habitat Loss	Development	Mainly NJDEP, Pinelands Commission, Public pressure on agencies. Public can protect snakes, leave habitat where possible on their properties.
	Forestry practices	Mainly NJDEP (Parks and Forests), Pinelands Commission, Public pressure on agencies, conservation organizations work to affect optimization for different sensitive species. Scientists of all stakeholder groups develop information on Pine Snake habitat needs to lobby Parks and Forests; public lobby for Pine Snakes. Conservation organizations and other publics can lobby for restrictions of off-road vehicles to reduce mortality.
	Infrastructure development	NJ Department of Transportation (DOT). NJDEP (Endangered Species and Nongame Project) influence DO T and work to build under-highway passages. NJDEP collect information on road-killed Pine Snakes to identify sensitive regions. Public can report Pine Snakes dead on the roads with their locations to the NJDEP database.
	Fire	Natural fires originally set back succession, providing open areas for Pine Snakes to nest and hibernate. Management of fires prevents the natural creation of open areas. State agencies (in collaboration with Pinelands Commission) can manage controlled burns (or forest cutting) to create open areas; conservationists and the public can lobby for creation of open areas, and can volunteer for such management actions.
Human Disturbance	Off-road vehicles	Conservation organizations, scientists, and the public pressure state and local officials, including NJDEP (ENSP [Endangered and Nongame Species Program], PF [Parks and Forests]) and law enforcement to manage off-road vehicles to reduce mortality on snakes and other wildlife, while providing for legitimate off-road recreational activity at levels which do not threaten natural resources.
	Poaching	NJDEP, law enforcement (both ENSP and PF) to monitor sensitive nesting and hibernation areas during peak activity times (spring, early summer nesting season, fall). Conservation organizations and private citizens to pressure government agencies and Pinelands Commission to enforce laws. Citizens can stop poachers when they see them, and raise awareness among neighbors about poaching.
Predators	Natural predators	Scientists from all stakeholder categories need to monitor natural predation rates to determine if actions by NJDEP are required. Public can report any incidences of predation on Pine Snakes to NJDEP database.
	Enhanced natural predators	Scientists from all stakeholder categories need to monitor whether there are increases in natural predators that are due to availability of food; state agencies, Pinelands Commission, and others conduct educational programs to explain the importance of not feeding animals, or leaving food available.
	Human commensals	NJDEP, Pinelands Commission and conservation organizations can educate the public about the threats from dogs and other pets to natural ecosystems, including snakes. All stakeholders need to make the effects of releasing pets into the wild known to the general public.
Prey Base	Population variations	NJDEP (ENSP and PF) and Pinelands Commission can fund and encourage studies on variations in prey populations, and the relationship to habitats and fragmentation. This information could be used to address habitat and development restrictions. To better provide prey for Pine Snakes, the public should not control rodents on undeveloped property that they own.
Management Needs	Lack of enforcement	NJDEP, law enforcement to ensure that personnel are used effectively to maximize protection during peak Pine Snake activity Periods. Conservation organizations and public to reinforce these needs. Public can report any infractions.
	Lack of key information	While NJDEP and Pinelands Commission require specific information on habitat needs and threats that pose a risk to populations, university scientists and other scientists have a responsibility to conduct studies to address specific needs. Public volunteers can help in monitoring, assessments, and conservation studies with time, money, and expertise. They can volunteer for research projects to allow long-term studies to continue.
	Lack of personnel and money	Conservation organizations and the public to lobby government agencies to devote more personnel and money to protection and conservation of Pine Snakes and other sensitive Pinelands Species. Industry and developers can set aside some funding for necessary assessments and monitoring of projects and mitigations to determine efficacy. Public can contribute to research and conservation projects.
	Education about Pine Snakes	All stakeholders can play a role in education, but public advocates (conservation organizations, Pinelands Commission) can continue to include Pine Snake conservation as part of their educational programs. All volunteers can educate their neighbors, friends, and family about the role of Pine Snakes and their threatened status in the state.

Fig. 7. Volunteers contribute directly to conservation efforts by helping to remove trees that are obstructing sun penetration to nests or hibernation sites (**Fig. 7a**), or taking data on snake behavior (**Fig. 7b**).

have shed (and we remain until they have emerged, dispersed, and are no longer visible; Fig. 8).

The number of NJDEP conservation officers and Park Police has declined, and numbers are inadequate to effectively cover all the areas that need to be patrolled for the range of species protected under their responsibility. Although there are key seasons for Pine Snake activity, some of the hotspots are not close together, making it more difficult to patrol them and apprehend poachers. Many of the nesting areas have been known for many decades, and poachers regularly check them, including putting out "sucker boards" for snakes to hide under (where they can readily find them to poach).

Conflicts among stakeholder groups: There can be conflicts among stakeholder groups, even among state agencies, and these should be acknowledged (Young et al. 2013). The Department of Environmental Protection has a number of divisions that have different mandates with respect to habitats and the animals within them. For example, the Endangered and Nongame Species Program (ENSP) is charged with protection of all animal species, except for fish and game species. The Division of Parks and Forestry (PF) is charged with managing the forests, which can include cutting, special use permits, and other activities. In some cases the activities conflict with the protection of habitat for a species, such as Pine Snakes. Pine Snakes require open areas for nesting and for hibernation sites (Burger and Zappalorti 1986, 2011a), but these need to be close to suitable forest for foraging and summer dens (Burger and Zappalorti 1988b, 1989). Cutting large swaths of forest removes effective habitat, results in fragmentation, and churns up potential nesting areas. Pine Snakes do not nest in sugar sand, nor in sand with many dense roots, but prefer some roots from Hudsonia to stabilize the soil (Burger and Zappalorti 1986, 1988a). However, removal of small areas of trees can open the canopy and be optimal for Pine Snakes (Burger and Zappalorti 2011a), as well as for other snakes (Webb et al. 2005).

The pressures within each agency can also differ. For example with Pine Snakes, ENSP desires to keep off-road vehicles (ORVs) away from sensitive areas (nesting, hibernation) to avoid habitat destruction, and direct mortality, and would keep ORVs out of the forest during peak snake movement and activity periods (spring, nesting, fall). By contrast ORV users petition Parks and Forests to allow them to use ORVs in the forests at other times. Off road vehicle users have strong lobbying groups. Agency management is likely to listen to a vociferous group with many members. However, ORVs churn up nesting areas, killing eggs and hatchlings, and making habitat unusable for nesting, and they also unintentionally run over basking or moving snakes because large Pine Snakes are cryptic and invisible to a motorbike moving through narrow forest trails at excessive speeds (Burger et al. 2007).

Conclusions

Key contribution of stakeholders to conservation: Including a variety of stakeholders who have a strong interest in the conservation of a rare plant or wildlife species typically has a positive outcome. A good example of stakeholder cooperation was the planning and writing of a comprehensive management and recovery plan for the Gopher Tortoise (*Gopherus polyphemus*), which was subsequently listed as a state "threatened" species (Florida Fish and Wildlife Conservation Commission 2012). Input from expert Gopher Tortoise stakeholders provided their years of knowledge and experience which was included in the recovery and management plan (Ashton and Ashton 2008). This case, however, did not have as inclusive a group of stakeholders, including non-governmental agencies (NGOs) and the general public.

Our case study illustrates how a range of stakeholders can aid in research and conservation of Pine Snakes in a number of ways, and help ensure that long-term studies provide the information needed for their continued protection. The various stakeholders we cooperated with have contributed markedly to conserving Pine Snake

Table 3. Agencies and entities that directly contribute to research and conservation of Pine Snakes in New Jersey. The examples given relate to Pine Snakes and are used to provide an indication of the ways stakeholders can participate, having a positive or negative effect (+/-).

Type	Example	+/-	Contribution
Independent Scientist	Rutgers University, Other universities or colleges, museums	+	Design, oversee, and implement research and conservation on Pine Snakes, leading to publication in refereed literature and provision of information to the public. Train students, both graduate and undergraduate, and organize volunteers to participate in research projects (Burger et al. 1987, 1991; Burger 1989b, 1990, 1991a,b, 1998a,b, 2006; Burger and Gochfeld 1985; Rudolph et al. 2007; Miller et al. 2012.
Resource Agency	NJ Department of Environmental Protection (NJDEP), Endangered and Nongame Species Program	+	Responsible for listing species (endangered, threatened, species of special concern), and gathering information where needed to protect the species and enhance populations, if needed. Pine Snakes are listed as threatened in NJ, and the ENSP has had to respond to delisting calls by developers (the state prevailed). Lead evaluations of the status of all nongame species, and oversee and engage in research, including snakes (Burger and Zappalorti 1988a, b, 1989, 1992; Schwartz and Golden 2002; Golden and Jenkins 2003; Golden et al. 2009). NJDEP also bans ORVs on public lands (NJDEP 2002).
	NJDEP; Division of Parks and Forests	+	Responsible for administering NJ state parks and forests. Bass River State Forest and Wharton State Forest have been involved with actively preventing off-road vehicles on nesting and hibernation sites, and habitat manipulation to improve nesting habitat (Burger et al. 2007; Burger and Zappalorti 2011a, b).
	NJ Natural Heritage Program	+	Lists and catalogues all sightings of endangered, threatened, and special concern species. Information is useful to federal and state agencies, consultants, and others. Exact locations of Pine Snakes are not disclosed generally to other that state or federal agencies.
	Pinelands Commission of the Pinelands National Reserve	+	Responsible for administering the Pinelands National Reserve, including protecting habitat for threatened and endangered species, such as the Pine Snake (NJPC 2009).
Other Agency	Ocean County Department of Emergency Services	+	Provide facilities and office space for snake research (Burger and Zappalorti 1988).
Regulatory Agency	NJ Department of Environmental Conservation, Law enforcement	+	Responsible for enforcing state endangered species laws. Pine Snakes are heavily poached by snake collectors in some years (Burger and Zappalorti 2011a, b).
Conservation Organization	New Jersey Conservation Foundation	+	Major mission is the protection and conservation of NJ's species, populations, communities, and ecosystems. Engage in independent and collaborative research with Pine Snakes, protection of Pine Snakes on their properties, organizes volunteers to help with research projects. Provide funding where possible. Mobilize interest in conservation measures and influence protective laws and regulations. Provide expertise and volunteers to aid in conservation, such as placing barriers to ORV traffic on nesting and hibernation sites (Burger et al. 2007).
	Pineland Preservation Alliance	+	Dedicated to upholding the tenets of the (NJ) Pinelands Preservation Act, and protecting the plants and animals of the Pinelands; provides volunteers to assist in research and conservation projects, especially protecting sensitive areas from illegal off-road vehicle use.
	The Nature Conservancy	+	Work to conserve species and habitats; fund projects (Burger and Zappalorti 2015; Zappalorti et al. 2015).
	New Jersey Audubon	+	Provide volunteers to assist in research and conservation projects.
Other Non-governmental agencies	Outdoor hiking clubs: Burlington County Naturalists, Batona Trail Club	+/-	Report sightings of rare species, assist with filling in knowledge gaps in distribution for rare species.
Environmental Justice Communities	Some retirement communities	+/-	Some retirement communities are on low/fixed incomes; some retirees fear snakes, do not protect them, and kill them on sight; dogs can become predators. The original residents of the Pine Barrens ("Pineys"), who had small farms in the pines, protected Pine Snakes because they eat rats and mice. They left places for them to nest at the edges of fields (Burger and Zappalorti 2011a).
Public	Naturalists	+	Gather information, produce reports and books about animals or habitats (field guides; Conant and Collins 1998; Boyd 1991).

Table 3 (continued). Agencies and entities that directly contribute to research and conservation of Pine Snakes in New Jersey. The examples given relate to Pine Snakes and are used to provide an indication of the ways stakeholders can participate, having a positive or negative effect (+/-).

Type	Example	+/-	Contribution
	Conservationists, hunters.	+/-	Volunteer to help with research projects, help build hibernacula and collect data on life history characteristics. Help monitor populations (Gerald et al. 2006a, b). Hunters maintained hunting lodges in the Pines, keeping open areas around their lodges which are used by Pine Snakes for nesting and hibernation sites.
	Buck Run Hunt Club, Burrs Mill Hunt Club	+	Provide access and volunteers to help with research and conservation of Pine Snakes. Help build hibernacula and provide information on nesting sites and timing of nesting. Maintain open nesting areas for snakes (Burger and Zappalorti 1986, 1991; Zappalorti and Burger 1986; Burger et al. 1988).
	Other recreationists	+/-	Hikers, photographers, and others that walk through the Pine Barrens forests or roads. Usually protective of snakes, but may inadvertently kill or injure snakes. All foot and vehicular traffic within the pines can kill or injure snakes, and carry invasive seeds, leading to habitat changes.
	Retirement communities	+/-	Some retirees are protective of Pine Snakes, while others are afraid, and discourage, injure, or kill them.
	Traffic	-	There is significant mortality on paved roads, and on the sand roads that pass through the forest. Some people aim their cars toward the snakes, deliberately killing them (Himes et al. 2002; Golden et al. 2009).
	Off-road vehicle enthusiasts	-	Some recreationists (ORVs) make trails in the pines or on nesting areas, disrupting nests and killing snakes or destroying the underground nests (running over them; Burger et al. 2007).
	Snake enthusiasts and poachers	+/-	Snake enthusiasts help protect snakes and contribute time and money to snake research and conservation. Poachers can be a problem (poaching of nests averaged 29%/year, but was as high as 40%, Burger et al. 1992).
Consultants	Companies and scientists	+/-	Professionals that bid for work from state agencies and industry to census, monitor, or study species. Also conduct un-paid scientific studies. Contract work for the state always provides useful information (Zappalorti and Burger 1986; Zappalorti et al. 2014, 2015).
	Herpetological Associates	+	Consulting firm dedicated to providing sound scientific information to agencies, conservation organizations, and industry about amphibians and reptiles. Also conducts independent herpetological research (Zappalorti and Burger 1986; Burger and Zappalorti 2011a).
Industry	Varied	+/-	Provide funding for studies on their lands that they wish to develop; such funding results in information on nesting, hibernation sites, movement, and activity ranges (Gerald et al. 2006a, b).
Developers	General contractors	+/-	If in appropriate habitat, need to conduct an assessment of Pine Snake presence and abundance, depending upon contractor can be positive or negative; can produce important information on Pine Snakes (Zappalorti et al. 2015; Burger and Zappalorti 2011a), or can census at the wrong times or with the wrong methods.
	Builders Association of NJ	-/+	Challenged the threatened status of Pine Snakes; request delisting of rare species. Provide funding for state-required threatened or endangered species studies on proposed development site (Golden et al. 2009).

populations in New Jersey. They did so by volunteering to aid with research and conservation projects, educating the public about the role and importance of Pine Snakes in the Pinelands ecosystem, aiding in enforcement of laws and regulations, and providing funds for specific research tasks. For example, volunteers helped our research by searching for nest sites, and aiding with hibernation and radio-tracking studies. They greatly aided conservation efforts by cutting small groups of trees to provide open nesting habitat, removing herbaceous cover to increase the suitability of nesting areas, and adding logs to provide hiding places for hatchlings (Fig. 7). We note in passing that our project started before Pine Snakes were listed as a threatened species by the State of New Jersey, and it was our data (aided by stakeholders) that contributed to their listing.

We suggest that other herpetological studies can be greatly improved with the inclusion of stakeholders (Fig. 9). Each stakeholder group has the potential to contribute in many ways. State and county governmental agencies should be encouraged to enact laws and regulations to provide protection for herpetological communities, as well as to provide surveillance and law enforcement. The involvement of state agencies and NGOs has persuaded landowners to allow researchers to conduct studies on their land, and to consider easements or the purchase of land to provide wildlife corridors in connecting critical habitats. Land managers, either government agency, NGO, or private interests have directly aided in targeted conservation activities. In doing so they became aware of partnerships in field conservation to improve habitat (e.g., removal of vegetation or invasive species), prevent

Figure 8. Several Pine Snake females often nest in the same nest. Here we (R. Zappalorti and J. Burger) have removed four clutches (note they are bound together, making it possible to identify the eggs of three different females). Once females lay eggs, they exude a liquid that binds the eggs together. This partly prevents other females from disrupting the clutch and accidentally removing them to the outside while they are digging their own side chambers.

ORV entry (adding fencing, building berms, or other barriers), or educate the public about the importance of protecting Pine Snakes within their ecosystems.

NGOs can disseminate information through newsletters and programs on conservation needs, solicit volunteers from their organizations, and encourage contributions of money, equipment and time. Indirectly NGOs can advocate for state and local government to enact protection measures (laws, regulations), and provide conservation officers. By their example, NGOs can demonstrate the criticality of conservation for endangered or threatened species.

Many other organizations and individuals can also directly contribute to conservation of reptiles. For example, companies can provide volunteers and educate their employees about the importance of a range of species. Awareness of the plight of reptiles might result in managers altering the timing of activities (e.g., reduction of activity during critical nesting periods), and enhancement of vigilance throughout the year to avoid unnecessary harm. Companies can also develop a culture of ongoing contributions of research funds or volunteer assistance with field research and conservation.

Individuals can volunteer to aid projects, provide funding for projects, advocate at local, state and federal levels to protect reptile communities, and provide local information not necessarily known by others. Some people have historical knowledge of populations, nest and hibernation sites used, and changes in predator (or prey) abundance in a particular habitat. In one particular example, the site engineer at a hazardous material cleanup site became aware of both gestating, state-endangered female Timber Rattlesnakes (*Crotalus horridus*) and nesting Pine Snakes, and mentioned their presence to an adjacent non-profit conservation landowner. An innovative approach to enhancing the rattlesnake gestation and Pine Snake nesting sites was developed and implemented as part of the hazardous material cleanup. A permit was obtained for this new plan, and it was actually less expensive than the original remediation plan which would have ruined the gestation and nesting areas with unnecessary tree plantings.

In all the above examples, individuals are key. People working for governmental agencies, NGOs, businesses, and other organizations, as well as volunteers, can all contribute to advancing research and conservation of reptiles.

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Figure 9. Sometimes many volunteers are necessary for a project, either digging up a hibernation site (**Fig 9a**), clearing open areas for sun penetration, or digging up an old septic line to prevent collapses and injuries to snakes.

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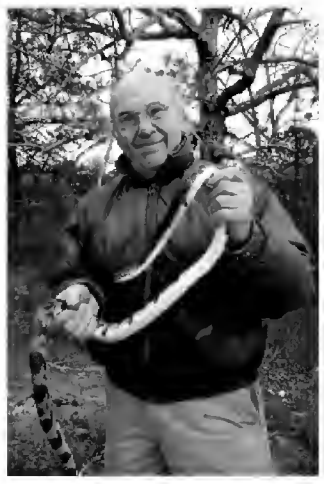

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Reproductive biology of *Tylototriton yangi* (Urodela: Salamandridae), with suggestions on its conservation

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***Abstract.*—Despite the long-term establishment and the species richness of the knobby newt genus *Tylototriton*, taxonomy of its members remained controversial, and little is known about the reproductive biology of its members, especially about their courtship behavior. Here we provide information on the reproductive biology of the Tiannan Knobby Newt, *T. yangi*, including the pre-spermatophore-deposition courtship behavior both in the field and in captivity, morphology of its eggs and larvae, and breeding habitat at the type locality. We compare different aspects of the reproductive biology interspecifically within the *T. verrucosus* group, and provide suggestions for future behavioral studies. In addition, with information about the reproductive biology of the species, we offer recommendations for its conservation accordingly.**

Keywords. Comparative ethology, courtship behavior, development, habitat, larvae morphology, sexual isolation

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Introduction

Although most biologists embrace the evolutionary species concept, wherein a species is defined as an independent evolutionary lineage, species delimitation can be difficult in practice using standard morphological and molecular approaches, especially for organisms with conservative morphologies and complex evolutionary histories (Sites and Marshall 2004; Marshall et al. 2006; Barley et al. 2013). The knobby newts of the genus *Tylototriton* Anderson, 1871 represent a classic example of such a challenging species-complex. Despite the establishment of the genus *Tylototriton* for more than a century, the species boundary of its type species, *Tylototriton verrucosus* Anderson, 1871, remains controversial to date, mostly due to the unsettled issue regarding its type specimens (Nussbaum et al. 1995; Chanda et al. 2000; Nishikawa et al. 2013, 2014; Phimmachak et al. 2015). As a consequence, species boundaries and taxonomic validity of remaining members of the *T. verrucosus* group remain unclear (Nishikawa et al. 2013, 2014; Phimmachak et al. 2015).

In contrast to the traditional morphological approach, ecological and ethological approaches, which examine reproductive ecology and courtship behavior, may provide additional evidence to delimit species boundaries and reveal insights into the evolutionary histories of organisms (Töpfer-Hofmann et al. 2000; Rundle and Nosil 2005; Marshall et al. 2006). In salamanders, courtship behavior patterns and pheromones used during courtship are known to be species-specific, and differences in courtship behavior and courtship chemicals can lead to sexual isolation among sympatric species, as well as among conspecific but allopatric populations (Verrell and Mabry 2003; Rissler and Apodaca 2007). Therefore, assessing behavioral differences during courtship among congeners of the genus *Tylototriton* may provide critical insights on its complex systematics and taxonomy.

However, much information on the reproductive biology, including courtship behavior, is lacking for many members of the genus *Tylototriton*, particularly species that were recently described (Nishikawa et al. 2015; Hernandez 2016). One such example is the Tiannan Knobby Newt, *Tylototriton yangi* (Hou, Li, Lv, 2012). First described by Hou et al. (2012) from the *T. verrucosus* group, limited detailed information was known regarding its typical habitat and reproductive ecology since its original description (Fei et al. 2012; Hernandez 2016).

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Fig. 1. Location of the study site (the type locality of *Tylototriton yangi*) at Gejiu, Honghe Prefecture, Yunnan Province, PR China. Numbered locations of potential breeding pools (abbreviated as PBP) are shown in yellow.

Understanding the reproductive biology of *T. yangi* in a comparative framework will facilitate future studies to investigate the evolution of reproductive biology of the genus. Furthermore, since the known distribution range of *T. yangi* overlaps greatly with that of major tin-mining sites in China, it is imperative that we understand its habitat requirements and reproductive biology so that effective conservation efforts can be developed and applied.

Here we provide detailed descriptions of the breeding habitats, pre-spermatophore courtship behavior both in the field and captivity, and morphology of eggs and larvae of the Tiannan Knobby Newt, *T. yangi*. In addition, we compare our descriptions to those available for other species in the *T. verrucosus* group, provide directions for future behavioral and ecological studies of the species group, and suggest conservation strategies.

Materials and Methods

Field observations

Field observations were conducted at the type locality of *T. yangi* in mixed plantations near Gejiu, Honghe Prefecture, southern Yunnan Province, from May 16th to May 18th, and from May 27th to May 28th 2014 (Fig. 1). Detailed locality information is not provided here to prevent potential poaching. Potential breeding pools (PBP) were located and surveyed twice during each day (first during the day, second from dusk until midnight). Plants and other animals around and within the PBPs were collected and photographed. These samples were later identified to species after fieldwork. Behavioral observations and recordings were made at night when the newts were active. Behavior patterns were recorded using a Nikon D7000 digital camera.

Observations in captivity

Five males and five females of *T. yangi* were collected from areas around Gejiu and Mengzi of Honghe Prefecture, Yunnan, China on May 28th. Collecting permits were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences, and animal care followed the Animal Welfare Protocol of Kunming Institute of Zoology, Chinese Academy of Sciences. Sexes were separated and housed in same-sex groups in four 60 × 30 × 40 cm plastic containers with five cm of water and live aquatic plants. Newts were fed live bloodworms and were allowed to acclimate to the captive environment for four days prior to the staging of heterosexual encounters. For the heterosexual encounters, two trials, with two replications each, were conducted at different water depth to determine whether water depth influences courtship behavior. For the first trial, two active males and one of the largest females were placed in a circular plastic container (diameter one m) filled with 15 cm of water and observed at 1 a.m. on June 5th and again on June 6th. All interactions among individuals were observed for 60 minutes, and courtship behavior patterns were recorded using a Nikon D7000 digital camera. For the second trial, the same animals were placed into the same plastic containers with only five cm of water observated at 1 a.m.

Fig. 2. Habitat in which *Tylototriton yangi* was found at the type locality of Gejiu. Examples of typical breeding pools are shown at the right corner (from left to right, PBP#17 and PBP#12), and positions of other pools are indicated by white arrows. *Photographs by Kai WANG.*

on both June 5th and June 6th. Pre-spermatophore deposition courtship behavior patterns were recorded using the same equipment as in the first trial. After the observation sessions, all adults were released back to the wild.

Eggs and larval morphology

Embryos produced by females in captivity were maintained until hatching. Larvae were fed with live bloodworms and housed in five plastic containers. Photographs were taken at different developmental stages until larvae completed metamorphosis. Juveniles were kept for one week after metamorphosis and then released into the wild at the type locality.

Results

Breeding habitat

The dominant habitat type was secondary mixed forest with scattered water sources. Seventeen potential breeding pools were located around a reservoir, including one natural pool along a stream (potential breeding pool number 5, abbreviated as PBP#5) and sixteen artificial irrigation pools for agriculture (PBP#1–4, PBP#6–17) (Fig. 1). The irrigation pools were scattered along the forest edge in mix-crop plantations, and most pools were shallow (water depth from 5–30 cm, the deepest one, PBP#14, 90 cm) with aquatic vegetation. Shores of the pools consisted of either rocky walls with crevices or dense terrestrial vegetation (Fig. 2). No newts were found in the reservoir, moving streams, or pools that were connected to streams (PBP#5). In addition, no newts were found in the mining sediment pools or pools close to the tin mining site (PBP#2). These same habitats were occupied by other amphibian species, including *Aquixalus* sp., *Dianrana pleuraden*, *Duttaphrynus melanostictus*, and *Kaloula verrucosa*. In addition, loaches (*Misgurnus anguillicaudatus*) were found in some pools (PBP#14, 16, and 17).

Field behavioral observations

Six males and one female of *T. yangi* were observed after dusk from 20.00h May 17th to 01.00h the next day, in which all males were found at the bottom of irrigation pools of plantations (one in PBP#11, one in PBP#12, and four in PBP#13), while a female was found crossing the newly plowed plantation not far from pool #13. No behavior patterns that might be interpreted as territorial or aggressive (such as biting or chasing) were observed among males in pool #13; and interactions were limited to nudging (and perhaps sniffing) one another's snouts and bodies. After placing the female into pool #13, the closest male soon approached her and made several brief contacts with his snout to her head. He then moved forward to a position in front of the female, coiling his body into a "C"-shape and holding it next to his body. The female showed no interest and moved away (Fig. 3).

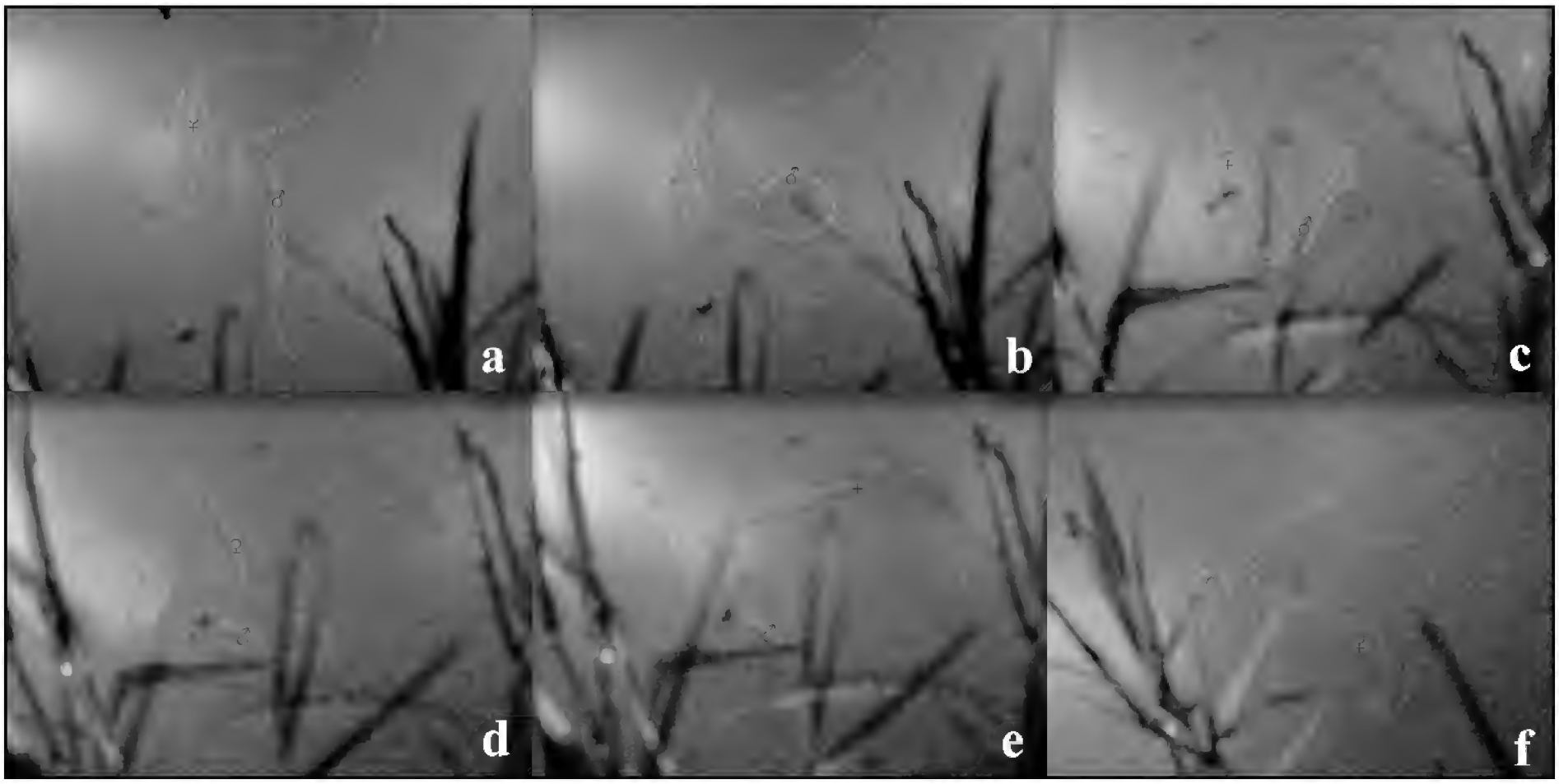

Fig. 3. Heterosexual encounters of *Tylototriton yangi* in the breeding pools near Yangjiatian Reservior, Gejiu, Yunnan Province, China. Clockwise from top left: **a)** male approaching a much larger female; **b)** male following the female; **c)** male coiling up and blocking female's path; **d)** male folding its tail toward the female; **e)** female swimming away; **f)** male following. *Photographs by Kai WANG.*

Another seven males were observed at night from May 27th to May 28th (two in pool#11, one in #13, and four in #14), all of which were on the substrate in water and not on land, and five larvae were found in pool #17.

Captive behavioral observations

As with all newts, sperm transfer in *Tylototriton* is accomplished by means of a spermatophore, placed on the substrate by the male and then is taken up into the cloaca of the female (Houck and Arnold 2003). Pre-spermatophore deposition courtship behavior patterns were identical to those observed in the field, and were the same for the two captive trials despite differences in water depth. Males were not observed to clasp females in amplexus. Here we provide an ethogram of the behavior patterns observed before spermatophore deposition in our two-males/one female trios (actual deposition was not observed) (Fig. 4).

(1) Swim away: the female turns or moves away from an approaching male.
(2) Nudging among males: males get distracted by other males' movements and nudge (sniff?) the head and lateral body of other males; but they quickly lose interest and move away from each other.
(3) Follow: the male rapidly moves after the female as she moves away from him.
(4) Approach: the males move toward the female when she is stationary.
(5) Male touch: the male makes repeated contacts with his head to the female's head, lateral body, especially her orange warts, and the lateral aspect of the proximal portion of her tail.
(6) Female nudge: with the pair in close proximity, the female turns her head toward the male and nudges (sniffs?) him with her snout.
(7) Male rub: the male repeatedly rubs his snout and cheek horizontally and laterally on the head and lateral aspect of the female's body, especially her orange warts.
(8) Tail tremble: the female trembles her tail when the male rubs her body with his cheek.
(9) Tail fan: the male moves forward and turns to place his body in front of the female. The male then curls the posterior part of his body and folds his tail inward in a "S"-shaped posture, with the tip of his tail is close to its base. He then rapidly undulates or fans the distal portion of his tail laterally in a fluid movement toward the female for 3–4s.

Eggs and larval morphology

Eggs were laid individually, not adhered to plants, on the floor of the container, or to one another, even though alternative oviposition materials were available in the containers. The animal pole was dark and the vegetal pole was white (Fig. 5a), and cleavage was observed in most embryos about 24 hours after their initial discovery. Since different sexes were kept separately except during the heterosexual encounter trials, and no actual mating occurred during the heterosexual encounters, females must have mated and so acquired sperm in the field prior to capture. At room temperature (20–25 °C), the hatchling period was 15 days.

Newly hatched larvae were between 10–12 mm in total length with large eyes; one pair of balancers was present on the lower aspect of the sides of the head; small

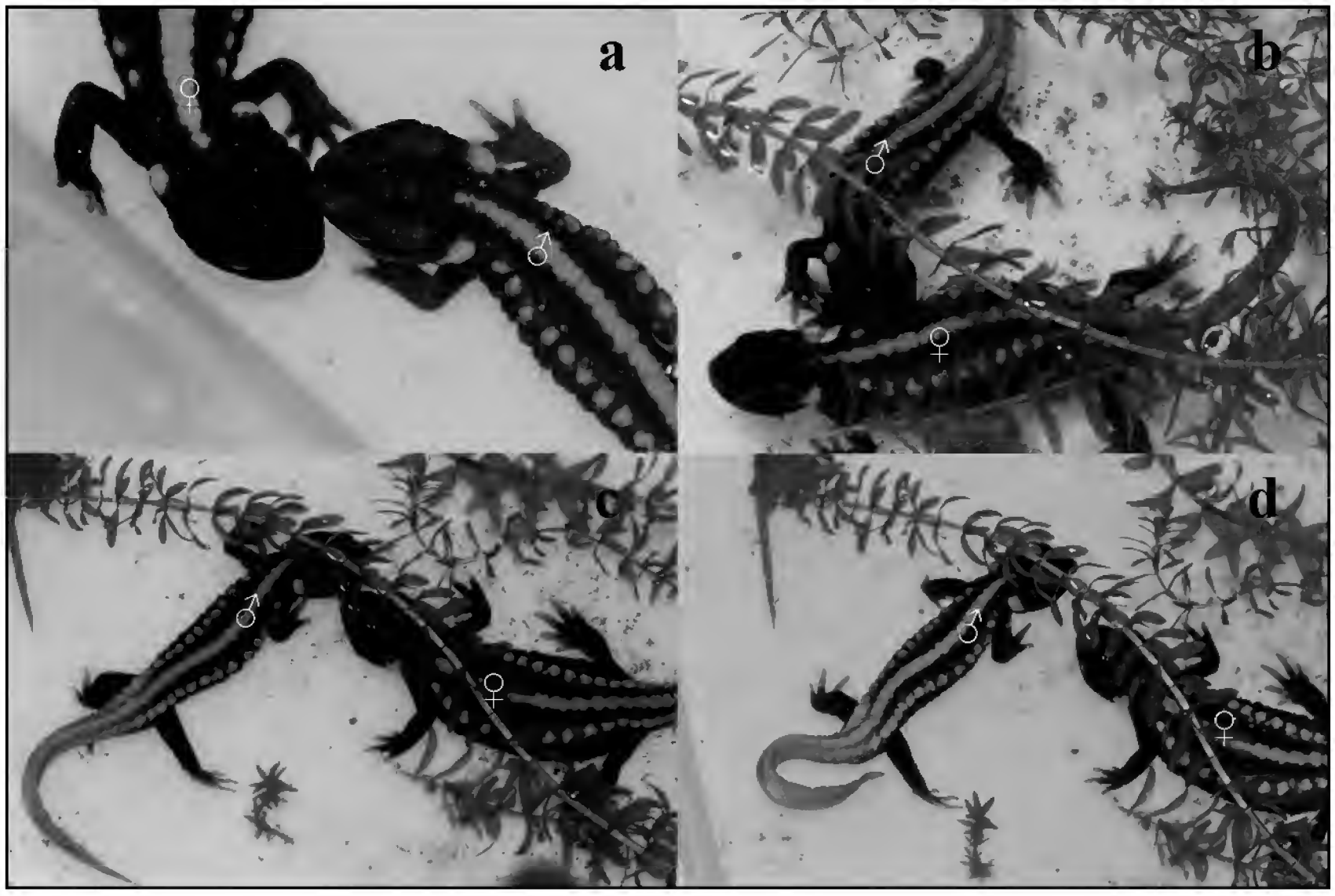

Fig. 4. Pre-spermatophore courtship behavior pattern of *Tylototriton yangi* in captivity. Clockwise from top-left: **a)** male nudging the side of the female's head with his snout; **b)** male nudging the side of the female's body; **c)** male blocking female's path and beginning to fold his tail; and **d)** male fanning the tip of his tail toward the female's head. *Photographs by Kai WANG.*

forelimb buds were present with very indistinctive toes; individuals had large abdominal yolk sacs; three pairs of gills were present, all of which were well-developed and were the same length as the head; tail fins were relatively deep (dorsal fin began from anterior part of the body, which runs for about three-fourths of the total length; ventral fin began from the posterior edge of the yolk sac, which runs about one-third of the total length). The dorsal surface of the body was yellowish brown and speckled with small dark dots, which formed two lateral bands running along the dorsal midline as well as the mid-lateral line. Speckled patterns also occurred on the tail fins. The gills were light pink and somewhat translucent, and the yolk sac was bright yellow with very few speckled patterns on the upper edges (Fig. 5b).

About five days after hatching, three toes showed on the distal end of the forelimbs and the tail fins were more developed (Fig. 5c). Through the development, the coloration of the larvae got darker, and the gills and the tail fin continued to grow. Later-stage larvae were brownish yellow with dark speckled patterns, possessed high tail fins and long gills, which were also speckled (Fig. 5d). Older pre-metamorphic larvae began to show some adult morphology, in which the head was less pointed, dorsal coloration became dark brown with developing light-colored patches along dorsolateral line, and the tail fins and gills were less translucent (Fig. 5e). Right before metamorphosis, larvae resembled adults in morphology: coloration became black, the head broadened and showed some trace of ridges, mid-dorsal orange ridge started to show, and a series of small orange warts became distinct dorsolaterally (Fig. 5f). Gills eventually disappeared, and the metamorphosis was completed in approximately 115 days (Fig. 5g).

Discussion

Review of courtship behaviors of Tylototriton verrucosus group

Significant differences in pre-spermatophore-deposition courtship behavior have been reported among different populations of *Tylototriton verrucosus sensu lato* from India (Roy and Mushahidunnabi 2001; Deuti and Hedge 2007), upper Myanmar (Boulenger 1920), southwest China (unpubl. data), and from the pet-trade with unknown locality (Sparreboom 2014). For the Indian populations, Roy and Mushahidunnabi (2001) reported that individual newts display extensive nose rubbing, tail fanning, and ventral amplexus (the male clasps the female's forelimbs with his forelimbs, with his dorsal side facing her ventral side). Similar amplexus behavior was also observed for the upper Myanmar population (Boulenger 1920). However, Sparreboom (1999, 2014) reported only tail fanning behavior in *T.* cf. *verrucosus* for pet-trade individuals from an unknown locality, and he did not observe extensive nose rubbing or ventral amplexus. For the topotypic individuals of *T. verru-*

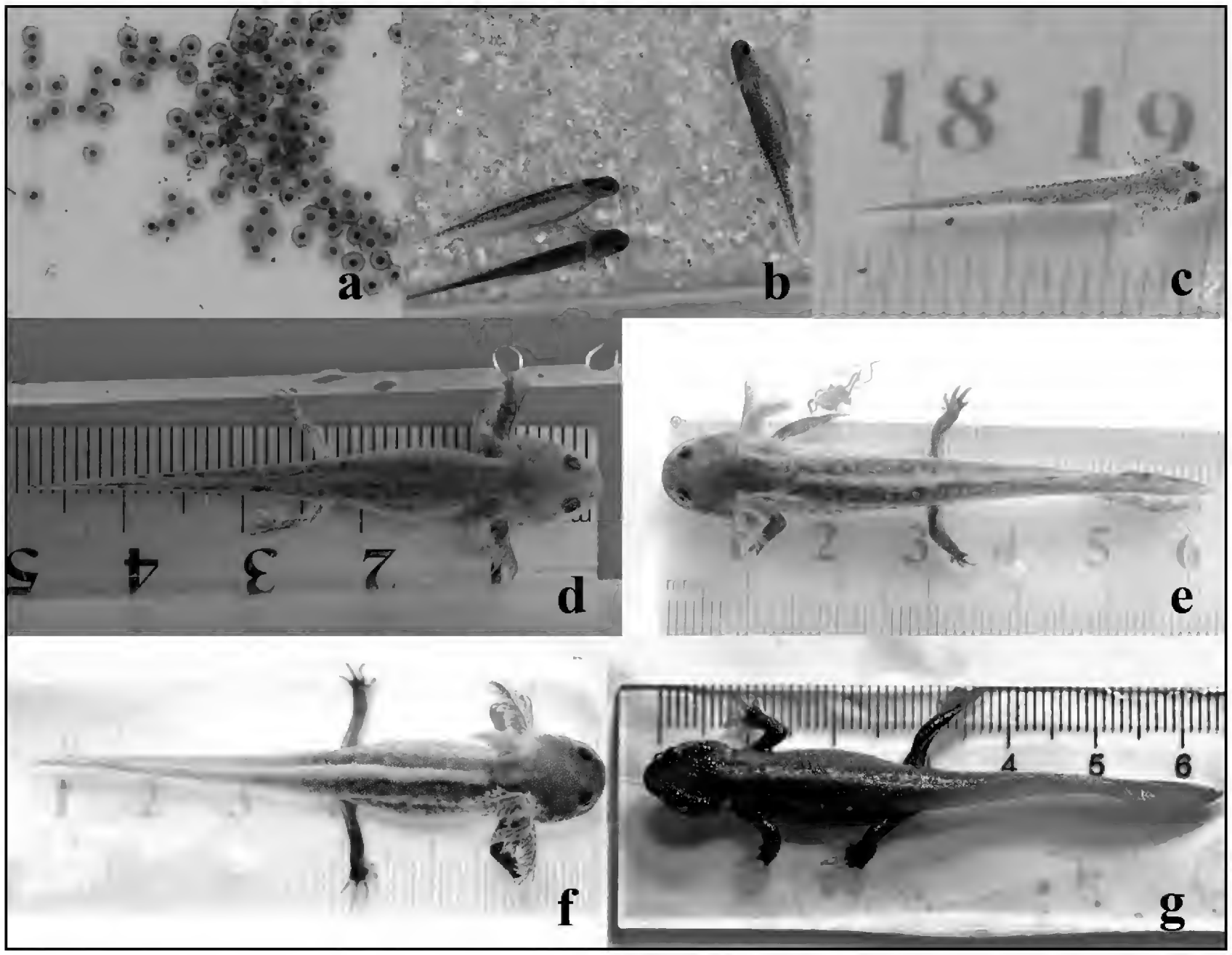

Fig. 5. Developmental series from fertilized embryos to newly metamorphosed juvenile of *Tylototriton yangi*. Clockwise from the upper left: **a)** fertilized embryos of *T. yangi*; embryos sank to the bottom of water, and were not adhesive to plants, the bottom of the container, or to one another; **b)** newly hatched larvae with one pair of balancers 6-day post-hatch; **c)** larva 17-days post-hatch, in which the forelimbs became visible; **d)** larva 50-days post-hatch; **e)** larva 75-days post-hatch; **f)** pre-metamorphic larva 95-days post hatch; **g)** newly metamorphosed individual 115-day post hatch. *Photographs by Kai WANG and Guangyu LI.*

cosus from southwestern Yunnan Province, China, Yuan observed nose-rubbing and tail-fanning behavior, but not ventral amplexus (unpubl. data).

Recently, several new species have been described from the *T. verrucosus* complex, including *T. himalayanus* from Nepal (Khatiwada et al. 2015) and *T. shanorum* from northern Myanmar (Nishikawa et al. 2014). Given the close geographic distance between the type localities of the two newly described species and the localities of previously identified *T.* cf. *verrucosus* populations with different courtship behaviors from India and Myanmar (Boulenger 1920; Roy and Mushahidunnabi 2001), differences in courtship behavior among these two populations may represent differential behaviors of *T. himalayanus* and *T. shanorum* respectively, and ventral amplexus may be a characteristic behavioral pattern that differentiates *T. himalayanus* and *T. shanorum* from *T. verrucosus sensu stricto*.

In contrast, Hernandez (2016) reported ventral amplexus during courtship in *T. verrucosus sensu stricto*. However, the reference Hernandez cited describes courtship behavior of *T. verrucosus* populations from Thailand (Humphrey and Bain 1990), which, based on Hernandez's book, are now considered as *T. uyenoi* Nishikawa, Khonsue, Pomchote, Matsui 2013, instead of *T. verrucosus sensu stricto*. Furthermore, the photographic evidence of ventral amplexus of *T. verrucosus sensu stricto* that Hernandez (2016) reported is of pet-trade individuals in France with no known locality information; and based on the external morphology of the individuals in the photo, these individuals should be identified as *T. shanorum*, as Hernandez suggested in his own book. Therefore, we recommend that further behavioral studies are needed to confirm the courtship behavior of *T. verrucosus sensu stricto* using topotypic individuals of the species.

Comparative reproductive biology of Tylototriton yangi

Based on our results, the reproductive biology of *Tylototriton yangi* differs substantially from what is known for other species of the *T. verrucosus* group, especially in terms of courtship behavior and egg morphology (Table 1). The courtship behavior of *T. yangi* is most similar to those of Indian populations of *T.* cf. *verrucosus*, in which they all court in water, exhibit tail-fanning movements, and display extensive nudging and rubbing behaviors

Table 1. Differential reproductive biology of members of the *Tylototriton verrucosus* group. -: absent; +: present.

		Courtship behavior displayed by males					Characteristics of eggs/ clutches	
Species	Source	Sniffing	Nose-rubbing	Tail fanning	Ventral amplexus	Courtship site	Eggs singular or forming clusters	Adhesive layer of eggs
Tylototriton yangi	Present study	+	+	+	-	Aquatic	Singular	-
Tylototriton shanjing	Ziegler et al. 2008; Li et al. 2012	+	-	+	-	Mainly Terrestrial	Singular, sometimes small clusters	+
Tylototriton cf. *verrucosus*	Boulenger 1920; Roy and Mushahidunnabi 2001; Deuti and Hedge 2007; Sparreboom 2014	+	+	+	+	Aquatic	Singular, sometimes small clusters	+
Tylototriton kweichowensis	Hu 1994; Tian et al. 1998	+	-	+	+	Aquatic	Singular	-
Tylototriton taliangensis	Fleck 1997; Fei et al. 2006; pers. comm.	+	-	+	+	Aquatic	Singular	-

(Roy and Mushahidunnabi 2001). However, the Indian population of *T.* cf. *verrucosus* displays ventral amplexus during its courtship (Roy and Mushahidunnabi 2001), which was not observed in the courtship of *T. yangi* in our study. Compared to populations of *T.* cf. *verrucosus* from the pet-trade with unknown localities, *Tylototriton yangi* displays extensive nose rubbing and nudging (sniffing?) behavior prior to tail fanning, which were not observed in pet-trade *T.* cf. *verrucosus* (Sparreboom 1999, 2014). In addition to differences in courtship behavior, *Tylototriton yangi* also differs from all populations of *T. verrucosus sensu lato* in egg morphology, in which eggs of *T. yangi* do not possess an adhesive outer layer, whereas those of the latter are adhesive and attached to aquatic vegetation (Roy and Mushahidunnabi 2001; Deuti and Hedge 2007; Wang, pers. observ.).

For other species, *Tylototriton yangi* differs from *T. shanjing* by courtship site (aquatic vs. mainly terrestrial), showing extensive nudging (sniffing?) and nose-rubbing behavior, and non-adhesive, singular eggs (vs. adhesive eggs sometimes in small clutches) (Ziegler et al. 2008; Li et al. 2012), and from *T. kweichowensis*, *T. taliangensis*, and *T. pseudoverrucosus* by showing extensive nose rubbing behavior and absence of ventral amplexus (Hu 1994; Fleck 1997; Tian et al. 1998; Fei et al. 2006; Hernandez 2016).

In contrast, recently Hernandez (2016) reported ventral amplexus during courtship in *T. yangi*, without references or photographic evidence, and he noted males of the species would develop rugose nuptial pads on their forelimbs during the breeding season, as in the amplectant salamandrid *Pleurodeles*. However, such amplexus behavior and the development of nuptial pads during breeding season were not observed during our field or captive observations. Further study is needed to confirm the presence of amplexus behavior in *T. yangi*.

Importance of chemical communication in courtship of Tylototriton

In newts and salamanders, olfactory signals are involved in intersexual recognition both within and among species (Dawley 1984, 1986). The extensive snout nudging and rubbing behavior patterns that we observed in male *T. yangi* suggests that they may obtain olfactory information from females during courtship: nudging may be sniffing. It may be that glands on the heads and in the warts of these newts show sexual dimorphism in glandular products, enabling discrimination between the sexes. On the other hand, Li et al. (2012) suggested that *T. shanjing* did not show any sniffing or nudging behavior and seemed to rely on visual cues at the beginning stage of courtship. Given these apparent differences in cues used in recognition processes among *Tylototriton* species and examples of behavioral isolation through chemical recognition in desmognathine salamanders (Tilley et al. 1990; Verrell and Mabry 2000; Mabry and Verrell 2004), it is possible that behavioral isolation also is present among species in the genus *Tylototriton*. Further work is needed to determine whether these behavioral differences, occurring before spermatophore deposition and at a time when species recognition might be expected to occur, result in decreased successes of heterospecific encounters (Verrell and Mabry 2003). Continued work on systematics and reproductive biology will surely reveal more about pattern and process in the evolutionary history of the genus *Tylototriton* generally, and the *T. verrucosus* group specifically.

Conservation of Tylototriton yangi

Our field observations indicate that scattered permanent ponds and other permanent bodies of stationary water are used for reproduction by *T. yangi*. Not all available water

Figure 6. Habitat destruction of *Tylototriton yangi* in southern Yunnan Province, China. **a)** Coal mining site at Yangjie, Mengzi, Yunnan Province, China; **b)** illegal tin mining at the type locality of *T. yangi* in Gejiu, Yunnan, China; **c)** Deforestation and infrastructure constructions at the type locality of *T. yangi* in Gejiu, Yunnan, China. *Photographs by Kai WANG.*

sources were occupied by newts during the duration of this study (e.g., the reservoir, and PBP#10, PBP#15, and PBP#16), and some pools (e.g., PBP#13 and PBP#14) were used by more newts than the others. These differences in pool use may be due to ecological factors such as nearby canopy coverage, amount of aquatic vegetation, water depth, food availability, and predation risk. We found the most newts in deep pools (30–50 cm in depth) with no large aquatic predators (e.g., large fish), some but not dense aquatic vegetation and dense surrounding terrestrial vegetation. These may be key factors for breeding site selection by *T. yangi*. Further studies are needed to determine the details of factors that affect breeding-site selection.

Having a restricted range in southern Yunnan Province of China, *Tylototriton yangi* faces a number of serious anthropogenic challenges. Habitat loss, especially of breeding habitat, is the greatest threat to the species (Hernandez 2016). Heavy tin/coal mining and accompanying deforestation were observed at our field sites during this study. This contaminated remaining potential breeding ponds and split terrestrial habitats into fragmented patches (Fig. 6). In addition to the habitat loss, illegal collections are the second most serious threats to the persistence of local populations of *T. yangi*. Local people harvest breeding adults from May to July every year, which are then dried and sold for traditional medicines. In addition, individuals are collected and sold alive as exotic pets in the illegal pet-trade. In fact, *T. yangi*, which was confused with *T. kweichowensis*, was the most common species of *Tylototriton* sold in the U.S. market before the official importation ban of Asian newts (Rowley et al. 2016), and illegally collected animals have also reached European countries such as France, Germany, and Russia (Hernandez 2016).

Because of these anthropogenic challenges, we recommend increasing attention to the conservation of the endemic species, *Tylototriton yangi*. Specifically, we recommend: 1) adding *T. yangi* to the List of Endangered Species of China as a Class II nationally protected species; 2) increasing law enforcement of the Wildlife Protection Act of China during the breeding season of the species from May to August, especially increasing patrol frequency in the pet markets and traditional medicine markets in Mengzi and Gejiu of Honghe Prefecture, Yunnan, China, 3) conserving existing adult habitats, particularly at the type locality in Gejiu, through restoration of natural plant communities and construction of artificial breeding ponds; and 4) initiating captive-breeding programs in research institutions in China, giving hope for subsequent release of newts to augment natural populations. Lastly, following the recommendation by Fei et al. (2012) and IUCN assessment criteria (extent of occurrence estimated to be < 20,000 km^2, severely fragmented, and inferred continued decline in extent of occurrence and area of occupancy), we recommend the listing of *T.*

yangi as Vulnerable under IUCN assessment criteria.

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Temperature sex determination, incubation duration, and hatchling sexual dimorphism in the Española Giant Tortoise (*Chelonoidis hoodensis*) of the Galápagos Islands

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***Abstract.*—Sex determination (SD) mode is documented in only 26% of turtle species; temperature dependent sex determination (TSD) is common but not ubiquitous. SD mode is documented for only five tortoise species; all of these have TSD with the la pattern. Temperature dependent sex determination was reported in Galápagos tortoises (*Chelonoidis nigra* complex) in 1991 based solely on a personal communication. Here we report TSD pattern, incubation duration, and hatchling sexual dimorphism in the Española Giant Tortoise (*Chelonoidis hoodensis*) of the Galápagos Islands based on experiments conducted in 1986–87. We found strong evidence for Type Ia TSD, a pivotal incubation temperature of 28.3 °C, and a range for transition temperatures of 25.2–31.4 °C. We also found longer incubation durations for male than for female hatchlings, and describe a new method for sex identification for hatchling tortoises. These results have important implications for incubation of eggs for head-starting captive breeding, especially for conservation purposes, and for interpretation of data from natural nests. We caution against the assumption that all *C. nigra* complex species have similar pivotal or transitional temperature ranges, and encourage evaluation of more species in this group.**

***Resumen.*—El modo de determinación sexual (DS) solamente se ha documentado para el 26% de las especies de tortugas; la determinación del sexo por la temperatura (DST) en las tortugas es común pero no es generalizada. Se conoce el modo SD solamente para cinco especies de tortugas; todas ellas tienen el modo de DST. Se reportó en 1991 la determinación TSD para las tortugas de Galápagos (complejo *Chelonoidis nigra*), sobre la base de una comunicación personal. En este trabajo reportamos el patrón de DST, la duración de la incubación y el dimorfismo sexual a la eclosión en *Chelonoidis hoodensis* (la Tortuga Gigante de Española de las Islas Galápagos), sobre la base de experimentos realizados entre 1986–87. Nosotros encontramos firme evidencia para el DST tipo Ia, una temperatura pivotal de incubación de 28.3 °C y un rango de temperaturas transicionales de 25.2–31.4 °C. También detectamos que los períodos de incubación hasta la eclosión de tortugas machos fueron más prolongados en comparación con las hembras. Estos resultados tienen implicaciones ventajosas e importantes para la incubación de los huevos y para la interpretación de datos tomados de nidos naturales. Sugerimos evitar el inferir que todas las especies del complejo *C. nigra* tengan rangos de temperaturas transicionales similares y sugerimos la evaluación de más especies dentro de este grupo.**

Keywords. Turtle, reproduction, egg, conservation, life history, husbandry

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Introduction

Sex determination (SD) mode is documented in only 86 (26%) of the approximately 335 known turtle species; temperature dependent sex determination (TSD) is common but is not ubiquitous (The Tree of Sex Consortium 2014a, b). In the family Testudinidae (tortoises, ca. 57 extant species, TTWG 2017), SD mode is documented for only five species: *Testudo hermanni* (Eendebak 1995), *T. graeca* (Pieau 1971), *Gopherus agassizii* (Spotila et al. 1994), *G. polyphemus* (Burke et al. 1996; Demuth 2001), and *Malacochersus tornieri* (Ewert et al. 2004); all have TSD Type Ia. Two other Testudinidae ("*Geochelone elephantopus*" = *Chelonoidis nigra* complex and "*G. gigantea*" = *Aldabrachelys gigantea*) were reported as TSD in Janzen and Paukstis (1991), however both reports were based on unpublished data. The source data for *C. nigra* complex was unclear but presumably based on unpublished work by Sancho (1988) (Janzen, pers. comm.).

Chelonoidis is the largest tortoise genus (ca. 15 extant species, TTWG 2017); all *Chelonoidis* species are South American and most (10–12) *Chelonoidis* species are in the *C. nigra* complex (Galápagos giant tortoises) (van Dijk et al. 2014; Poulakakis et al. 2015; TTWG 2017). Populations of Galápagos giant tortoises have been greatly reduced, in some cases to extinction, due to predation by humans and by interactions with introduced species (MacFarland et. al. 1974a, b). Captive rearing of several *Chelonoidis* species for repatriation to their islands of origin has been an important part of Galápagos conservation programs (Cayot et al. 1994; Cayot 2008). These programs have become increasingly sophisticated, now including genetic analyses (Russello et al. 2010; Milinkovitch et al. 2013) and studies of the impact that repatriations have on vegetation (Gibbs et al. 2008).

The discovery that sex is determined by incubation temperature in most turtles has been of interest to the coordinators of Galápagos giant tortoise conservation programs for decades. This is because detailed knowledge of the effects of incubation temperature on hatchling sex could help managers avoid obvious pitfalls, such as producing all males, and to deliberately manipulate sex ratios (Vogt 1994). However, SD studies of *Chelonoidis* have not progressed because sexually dimorphic characteristics typically take many years to develop and it is unacceptable to conduct risky procedures on individuals so valuable to conservation. Therefore, the development of quick, easy, and harmless ways to identify the sex of hatchlings (e.g., Burke et al. 1994; Mrosovsky et al. 1999; Valenzuela et al. 2004; Martínez-Silvestre et al. 2015) are potentially very valuable.

Typically, investigations of TSD target four parameters: 1) the TSD pattern (Ewert and Nelson 1991), 2) the pivotal (=threshold; Bull et al. 1982) temperature, (= the constant incubation temperature that results in 1:1 offspring sex ratios, Mrosovsky and Pieau 1991), 3) the transitional range of incubation temperatures (TRT) (= the range of constant incubation temperatures that produce both sexes), and 4) the temperature-sensitive period (TSP) (= portion of the incubation period during which incubation temperature can affect hatchling sex, Bull and Vogt 1981). We sought to identify the SD mode, pivotal temperature, and TRT of the Española Giant Tortoise (*Chelonoidis hoodensis*) of the Galápagos Islands and develop ways to identify hatchling sex using external morphology and incubation duration. This species has been the subject of long term conservation efforts (Gibbs et al. 2014). Española Giant Tortoises were reduced to just 15 individuals by 1960; these were brought into captivity 1963–1974 and became the parents of thousands of offspring (Cayot et al. 1994; Cayot 2008; Márquez et al. 1991). Nearly 1,500 offspring have been released onto Española, and successful reproduction was first observed starting in 1990 (Márquez et al. 1991; Cayot et al. 1994; Cayot 2008). Although *C. hoodensis* remains Critically Endangered (CITES I, IUCN Red List), this is clearly an example of a highly successful chelonian head-starting program, despite low levels of genetic variation (Milinkovitch et al. 2013).

Materials and Methods

Incubation of eggs at different temperatures

A total of 189 *Chelonoidis hoodensis* eggs laid in 1986 were incubated at three temperatures: 25.5, 29.5, and 33.5 °C (67 eggs at each temperature) at the Galápagos Rearing Center, Puerto Ayora, Santa Cruz, Galápagos, Ecuador. Eggs were placed in plastic boxes with damp vermiculite; boxes were covered and placed in incubation chambers at constant temperatures. Boxes were rotated inside the incubators once per week to avoid effects of any thermal gradients in the chamber (Gutzke and Paukstis 1983). Incubation data were also collected from six additional tortoise hatchlings incubated and hatched earlier in the same facility.

Sex identification

Hatchling sex was identified in three ways: by direct gross observation of gonads, histological examination of gonads, and by laparoscopy. The gonads from 35 young tortoises that died of natural causes were examined via both direct gross observations of gonads and histological examinations of gonads. In some cases, the gonads were removed and fixed soon after the tortoise's death. However, most samples came from tortoises that were preserved intact either in formalin or alcohol. The gonads were embedded in Paraplast, cut at 5 µm thickness and stained with Harris' Hematoxylin and Eosin yellow stains. The histological procedures are described in Sancho (1988). Samples from tortoises fixed in alcohol

produced very poor histological sections and the gonads could not be identified. Fixations in formalin was also poor, but the gonads could be identified (Sancho 1988).

Laparoscopies were performed on 15 additional young tortoises; using standard surgical techniques. A small incision was made in the inguinal pocket just anterior to tortoises' hind legs to permit examination of the gonads. After observation, the skin was sutured and bathed with an antiseptic solution. We also counted the number of large dorsal scales in the tails of these individuals.

We assessed SD mode and estimated both pivotal temperature and TRT using the program TSD 4.0.3 (Girondot 1999, 2012; Godfrey et al. 2003) as in Burke and Calichio (2014). This program uses a maximum likelihood approach with a rather simple mathematical equation to compare the fit of observed data to four different sex determination models (genotypic sex determination, TSD IA, IB, and II) and uses Akaike Information Criterion (AIC) to rank the different models by penalizing for more parameters. The minimum data requirement for the TSD 4.0.3 program is sex ratio data from at least two constant temperature incubation experiments in which both sexes are produced.

Results

The juvenile gonads of Chelonoidis hoodensis

We examined the tortoise gonads both macroscopically and histologically; there was complete agreement between sex identification according to the gross morphology and the histology of gonads (Sancho 1988). The characteristics of juvenile gonads in *C. hoodensis* were similar to those of other turtles (Gutzke and Bull 1986), they consisted of two parts, the cortex and the medulla. The testicles of the juvenile tortoises (of up to two years of age) were white cylindrical structures of 7 to 8 mm in length, located on the ventral surface of the kidney. Testicles had a uniform reticular pattern of vascularization and the cortex was thin. Males lacked Müellerian ducts (or oviducts). Ovaries in juvenile tortoises, in contrast, were longer, thicker and flatter than testicles (mean length 11 mm). Vascularization was restricted to the medulla and the cortex was thick. In females, sex identification was aided by the presence of Müellerian ducts.

Germ cells were found in the medulla of males and in the cortex of females (Sancho 1988). Germ cells were rounder and larger than the somatic cells of the gonads. In one individual, germ cells were found both in the cortex and the medulla; in this embryo sex was not yet determined.

Effect of the temperature of incubation on sex determination

For unknown reasons, many embryos died during early incubation and others died during the last stages of incubation or at the time of hatching. Ten of the 11 hatchlings (91% male, hatch rate = 16.4%) from eggs incubated at 25.5 °C were identified as males, one was a female. At 29.5 °C, 27 (hatch rate = 40.3%) tortoises hatched and survived. We were able to identify sex in only 15 of these. Five of the 15 sexable hatchlings from eggs incubated at 29.5 °C were identified as males, 10 were female (33% male). All of the eggs incubated at 33.5 °C died during early development.

Results of the Hill and logistic models (program TSD 4.0.3) were indistinguishable using AIC (both AIC values = 8.99, Akaike weights = 0.50, goodness of fit < 0.001). This is strong evidence for Type Ia TSD. The logistic model predicted a pivotal incubation temperature of 28.3 °C (S.E. = 0.24), and a range for transition temperatures (TRT) of 25.2 °C (S.E. = 0.56)–31.4 °C (S.E. = 0.55). The Hill model predicted a pivotal incubation temperature of 28.3 °C (S.E. = 0.25), and a range for transition temperatures (TRT) of 25.2 °C (S.E. = 0.24)–31.5 °C (S.E. = 0.29).

Incubation duration for male hatchlings ranged from 125–167 days ($\bar{x}$ = 141.7) and incubation duration for female hatchlings ranged from 111–122 (x = 118.9). Incubation duration for males was significantly longer than for females ($t = 4.24$, d.f. = 18, two tailed $P < 0.001$).

The number of large dorsal scales in the tails of hatchlings identified as males ranged from 4–7 (n = 10, $\bar{x}$ = 4.9), females ranged from 2–5 (n = 10, $\bar{x}$ = 3.7). Male hatchlings had significantly more large dorsal scales on their tails than did females ($t = 2.48$, d.f. = 18, two tailed $P = 0.023$).

Discussion

Our finding that the Española Giant Tortoise (*Chelonoidis hoodensis*) has TSD is not surprising because this was reported by Sancho (1988) and is well known by the managers in charge of the Galápagos Tortoise Captive Breeding Program (Marquez et al. 1999; Burke, pers. obs.). However, we have added considerable detail to previously vague reports, including the pivotal temperature and the range for transition temperatures. These findings can inform captive breeding programs and field studies. For example, this type of information has been used in other species to predict hatchling sex ratios in natural nests (Georges et al. 1994; Delmas et al. 2008; Grosse et al. 2014).

Our finding that eggs incubated at female-producing temperatures and eggs incubated at male-producing temperatures differed in incubation duration is also not surprising, because the negative correlation between incubation temperature and incubation duration is well known for many turtles (e.g., Yntema 1978; Mrosovsky and Yntema 1980; Booth 1998). However, although this knowledge is commonly used in studies of sea turtles (e.g., Mrosovsky et al. 1999) to predict sex ratios of natu-

ral nests, we could find no similar studies in other turtles. We suggest that incubation duration could be used more commonly to predict sex in both artificially incubated eggs and eggs incubated *in situ.*

We consider our results indicating that female *C. hoodensis* had fewer large scales on the dorsal aspects of their tails interesting but needing additional investigation, especially a standardization of the method of counting tail scales. If the number of tail scales is sexually dimorphic, this technique could provide an extremely convenient way to sex hatchlings, and could be potentially valuable to many studies. We point out that incubation temperature is known to affect many hatchling characteristics, such as survivorship, body size, locomotor performance, and growth (e.g., Janzen 1993; Roosenburg and Kelley 1996; Demuth 2001). In addition, Burke et al. (1994), Valenzuela et al. (2004), and Lubiana and Júnior (2009) found significant sexual dimorphisms in body size and shape in hatchling turtles, while tail length is commonly sexually dimorphic in turtles as well (e.g., Casale et al. 2005).

Our results on pivotal temperature, transitional temperatures, and incubation duration should not be assumed to be identical in other *Chelonoidis*, even other *C. nigra* complex species. Variation in TSD patterns can occur between closely related turtle species (Bull et al. 1982; Ewert et al. 1994; Ewert et al. 2004) and even within a species (Ewert et al. 2005).Because of the diverse nesting microhabitats used by *C. nigra* complex species (Burke, pers. obs.), there may be considerable diversity in pivotal temperatures, TRT, and TSP.

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Ana Sancho (1965–2009) was an Ecuadorian biologist with an MBA specialized in project management, dedicated her work to the conservation of biodiversity, particularly in the Galápagos Islands. One of her research projects showed the link between Galápagos giant tortoises' sex and their eggs' incubation temperature. Later on, as Fishing Officer for South America at the NGO Traffic, she researched and coordinated the publication of the Report of Fishery activities and Trade of Patagonian Toothfish, which was presented at the Commission for the Conservation of Antarctic Marine Living Resources; as well as the Report on Sea Cucumber Trade in the Galapagos Islands. Between 2004 and 2008, she worked as coordinator of the UNDP/GEF project for the Control of Invasive Species in the Galápagos Archipelago. Among her main achievements was the establishment of a trust fund to control invasive species of the archipelago, which raised over $15 million. Her last professional activity was as coordinator of the project for the Implementation of Early Warning Systems and Natural Risk Management in 2009. She published several books and was part of Ecuador's official delegations in conservation events around the world. Apart from her extraordinary professional legacy, her friends and family remember her for her love and determination.

William H. N. Gutzke was a well-known herpetologist who studied embryonic development and phenotypic plasticity of reptiles and amphibians at both Memphis State University and the University of Memphis. Bill completed his Ph.D. (1984) on the influence of environmental factors on eggs and hatchlings of painted turtles (*Chrysemys picta*) and did post-doctoral work with James Bull at the University of Texas. He subsequently published 30+ articles in scientific journals, mentored four Ph.D. students, two Master's students, and at least 60 undergraduates. Bill Gutzke passed away in 2004.

Howard L. Snell is a professor in the Biology Department of the University of New Mexico and Curator of the Herpetology Division of the Museum of Southwestern Biology, also at UNM. Howard and his wife Heidi started work in the Galápagos Islands as volunteers from the US Peace Corps at the Charles Darwin Research Station in 1977. They continued visiting the archipelago through 2004. Within that interval they were variously based at Colorado State University, Texas Christian University, and Memphis State University before settling at the University of New Mexico in 1986. Howard worked with the Charles Darwin Foundation / Research Station as Program Leader for Reptiles, Vice President for North America, Program Leader for Vertebrate Ecology & Monitoring, and Director of Science Programs.

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Diversity, threat, and conservation of reptiles from continental Ecuador

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***Abstract.*—Ecuador is one of the most reptile-diverse countries in the world, with 464 currently recognized species. Similar to other taxa, reptiles in Ecuador face important conservation challenges because of anthropogenic activities. Using distribution data of nearly 90% of the species of reptiles from continental Ecuador, as well as information on ecosystem protection status and anthropogenic activities, we present the first comprehensive quantitative study of reptile conservation in Ecuador. While species richness is higher in northwestern Ecuador and the central-northern Amazon, the conservation priority areas identified in this study also include the central Pacific coast, southwestern Ecuador, and the central-southern Amazon. Similar areas have been identified by previous studies as conservation gaps. Thus, our study reinforces the idea of protecting those areas to improve the conservation of biodiversity in continental Ecuador.**

Keywords. Conservation priority areas, endemism, importance, opportunity, species distribution models

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Introduction

Compared to other groups of terrestrial vertebrates, reptiles have been subject to relatively few conservation studies leading to the identification of either global or local threats. Similar to amphibians, some authors (e.g., Gibbons et al. 2000; Todd et al. 2010) conclude that reptiles face six significant threats at a global scale: habitat loss and degradation, introduced invasive species, pollution, disease, unsustainable use, and climate change; however, those studies are mostly descriptive and their sampling of taxa is poor. Only recently was the conservation of reptiles analyzed at a global scale. Based on a worldwide sample of 1,500 species (~14.6% of total), Böhm et al. (2013) concluded that nearly 20% of species of reptiles are threatened with extinction, whereas another 20% could not be evaluated because of lack of data (Data Deficient). Moreover, a recent global analysis of the distribution of terrestrial tetrapods including 99% of all species of reptiles revealed that reptiles are not as well represented as mammals and birds under current conservation schemes (Roll et al. 2017).

Tropical areas have been identified as facing the most dramatic rates of habitat loss, as well as having high percentages of threatened reptile species (Böhm et al. 2013). With an area of only 284,000 km^2, Ecuador is a tropical megadiverse country crossed by two biodiversity hotspots, Tumbes-Chocó-Magdalena and the Tropical Andes (Mittermeier et al. 2004; Myers et al. 2000). To date 464 species of reptiles have been recorded in Ecuador (Torres-Carvajal et al. 2017), which represents the highest reptile diversity in the world when considering species number per unit area. Nonetheless, a comprehensive, quantitative study of diversity and conservation of reptiles in Ecuador is lacking.

In this study, we generate species distribution models for nearly 90% of species of reptiles from continental Ecuador based on distribution data from collections and the literature to assess (i) general patterns of diversity and endemism, (ii) threats, and (iii) priority areas for their conservation.

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Materials and Methods

Data collection

We obtained locality data points for 406 species of reptiles from three local museum databases—Museo de Zoología at Pontificia Universidad Católica del Ecuador (QCAZ), Museo Ecuatoriano de Ciencias Naturales (MECN), Museo de Historia Natural Gustavo Orcés at Escuela Politécnica Nacional (MEPN)—, HerpNET, Global Biodiversity Information Facility (GBIF), as well as from the literature. We validated each data point in ArcMap v. 10.2 (ESRI 2013) and removed taxonomically incongruent records (e.g., localities along the Pacific coast for species known to occur exclusively east of the Andes). Duplicate points (for the same species), as well as points <2 km close to each other were also removed to avoid oversampling bias in the analyses.

Species distribution maps

We used Maxent, a technique based on the principle of maximum entropy, to construct species distribution models (SDMs) for those species ($n = 287$) with ≥ 10 locality data points (Elith et al. 2011; Phillips et al. 2006; Renner and Warton 2013). As predictor variables, we used species presence data (i.e., geographical coordinates) and bioclimatic variables from Worldclim 1.4 (http://www.worldclim.org), which are based on temperature and precipitation data at ~1 km^2 spatial resolution (Hijmans et al. 2005). After removing highly correlated ($r > 0.8$) variables, selected explanatory variables were Temperature Seasonality, Annual Precipitation, Precipitation Seasonality, and Minimum Temperature of Coldest Month. Additionally, we included the ombrothermic index, ombrothermic index of the driest bimonth, and the terrain ruggedness index, which have been used in previous studies of distributional patterns in the Andes (Killeen et al. 2007; Tovar et al. 2013). To construct the models, we set the convergence threshold to 0.00001, maximum iterations to 1,000, and the regularization parameter to 1. SDMs with AUC (Area Under Curve) values below 0.7 were discarded (Elith and Leathwick 2007). SDMs for those species with 5–9 locality data points were constructed in Bioclim (Busby 1991; type output: true/false). After removing highly correlated ($r > 0.8$) variables, selected explanatory variables were Annual Mean Temperature, Mean Diurnal Range, Temperature Seasonality, Maximum Temperature of Warmest Month, Minimum Temperature of Coldest Month, Annual Precipitation, Precipitation of Warmest Quarter, and Precipitation of Coldest Quarter.

The distribution of species with four localities ($n = 43$) and species with rejected SDMs (i.e., AUC < 0.7) was delimited with minimum convex polygons. For species with fewer than four localities ($n = 76$), a 1 km^2 buffer was constructed around their presence data points.

Conservation priority areas

To identify priority areas for the conservation of reptiles we employed the Toolbox developed by Ríos-Franco et al. (2013) for ArcMap. This method integrates three criteria—threat, importance, and opportunity. We used it to identify regions outside the National Protected Areas System (PANE for its initials in Spanish) with maximum threat and importance values that show opportunity to be considered as priority areas for the conservation of reptiles in continental Ecuador.

According to the threat criterion, those areas with human activities are the most vulnerable. We generated a raster file with values from 0 (non-threatened zones) to 1 (highly threatened zones) based on the results of a short survey to reptile experts that included questions on risks, distances and intensity of threats, such as roads, oil fields, mines, and human settlements (Appendix). Areas that are easy to access pose a major threat to species because they represent great opportunities for humans to exploit natural resources (Sanderson et al. 2002). For this reason, we also created a file with geographic information on human settlements, roads, navigable rivers and terrain slope. The toolbox calculates the access probability from each of these elements assuming that a single person walks at a maximum speed of three km/h on a flat terrain without road access (Ríos-Franco et al. 2013).

The importance criterion prioritizes areas based on richness, endemism, and threatened species and ecosystems. We generated richness, endemism, and threat maps by overlapping the distributions of (i) all species of reptiles included in this study (see Species distribution maps above), (ii) endemic species, and (iii) threatened species. Details on the threat status of the reptiles from Ecuador will be published elsewhere. To identify threatened ecosystems, we generated a raster file with values between 0 and 1, where values close to 1 correspond to natural ecosystems that are well represented within the PANE, and values close to 0 correspond to the opposite (i.e., threatened ecosystems). The importance criterion was summarized in a raster file with values of 0–1, where values close to 1 represent areas with high levels of species richness, endemism, threatened species, and threatened ecosystems.

The opportunity criterion identifies areas with potential to be established as areas of conservation priority. Since 2008 the Ecuadorian government established the "Socio Bosque" program (SBP) to pay farmers and indigenous communities that voluntarily protect their native forests. We overlapped the threat and importance raster files with an "opportunity" file containing SBP areas, as well as private reserves and remnant vegetation.

Results

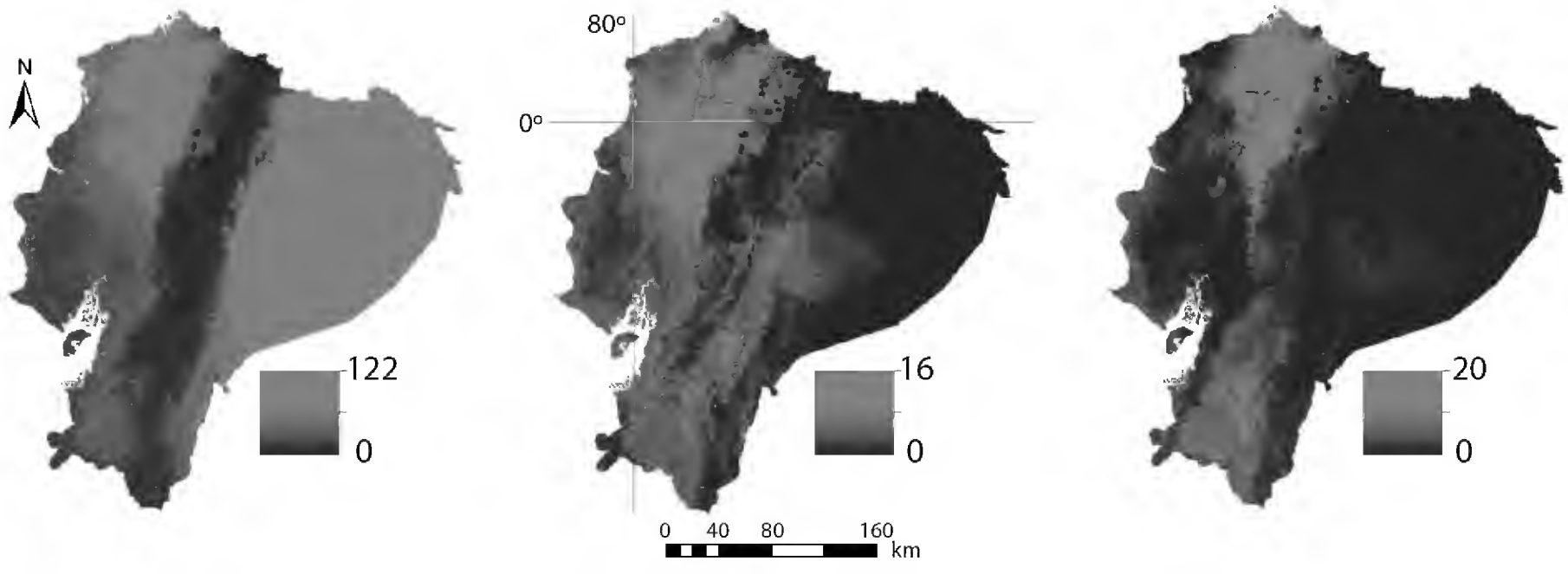

Figure 1. Maps of richness (left), endemism (center), and threat (right) for species of reptiles from continental Ecuador. Gradient values correspond to number of species.

Species richness, endemism and threat

Two regions in continental Ecuador have the highest numbers of species of reptiles. The most diverse region includes the central and northern Amazonian territories; however, northwestern Ecuador—Chocó and adjacent Andean slopes—is highly diverse as well (Fig. 1). Endemism is mostly concentrated in northwestern Ecuador, with large numbers of endemic species also present both on western and eastern Andean slopes. Similarly, the highest numbers of threatened species occur in northwestern Ecuador, followed by the Andes in southern Ecuador (Fig. 1).

Areas of conservation priority

The Pacific lowlands are more accessible to humans than any other regions in continental Ecuador. In contrast, according to the threat criterion, human activities that threaten reptiles are widespread mostly along the Andes and adjacent lowlands, with a slightly higher concentration in southern Ecuador (Fig. 2). The areas selected by the importance criterion based on species richness, endemism, and threat are described above; regarding threatened ecosystems, a large part of the Pacific lowlands, as well as Andean slopes in southern Ecuador are the least represented by the PANE. The central and southern Amazon include the areas with the greatest potential to be established as areas of conservation priority, most of them represented by SBP forests (Fig. 2).

Conservation priority areas were selected based on three of 12 possible solutions (Table 1). Accordingly, four areas were identified as the most important for the conservation of reptiles in continental Ecuador (Fig. 3): (1) the northwestern slopes of the Andes in Pichincha and Santo Domingo de los Tsáchilas provinces that include the Mindo-Nambillo Protected Forest, remnant Toachi-Pilatón vegetation, and SBP forest; (2) a central-south Amazonian area mostly in Morona Santiago province that includes remnant vegetation within the Kutuku and Shaimi cordilleras and SBP forest; (3) the southern

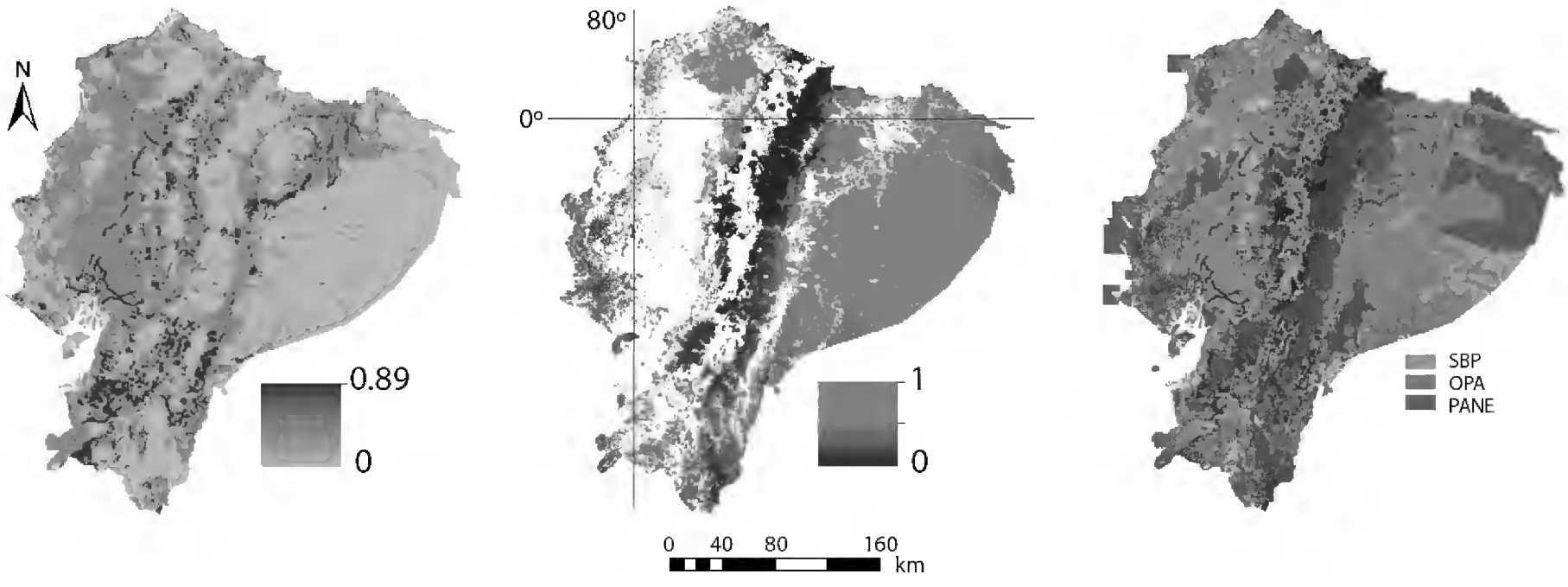

Figure 2. Maps of anthropogenic threat (left), importance (center), and opportunity (right), the three criteria used in this study to identify priority areas for the conservation of reptiles in continental Ecuador. SBP = Socio-Bosque protected forest, OPA = Other protected areas, PANE = National Protected Areas System.

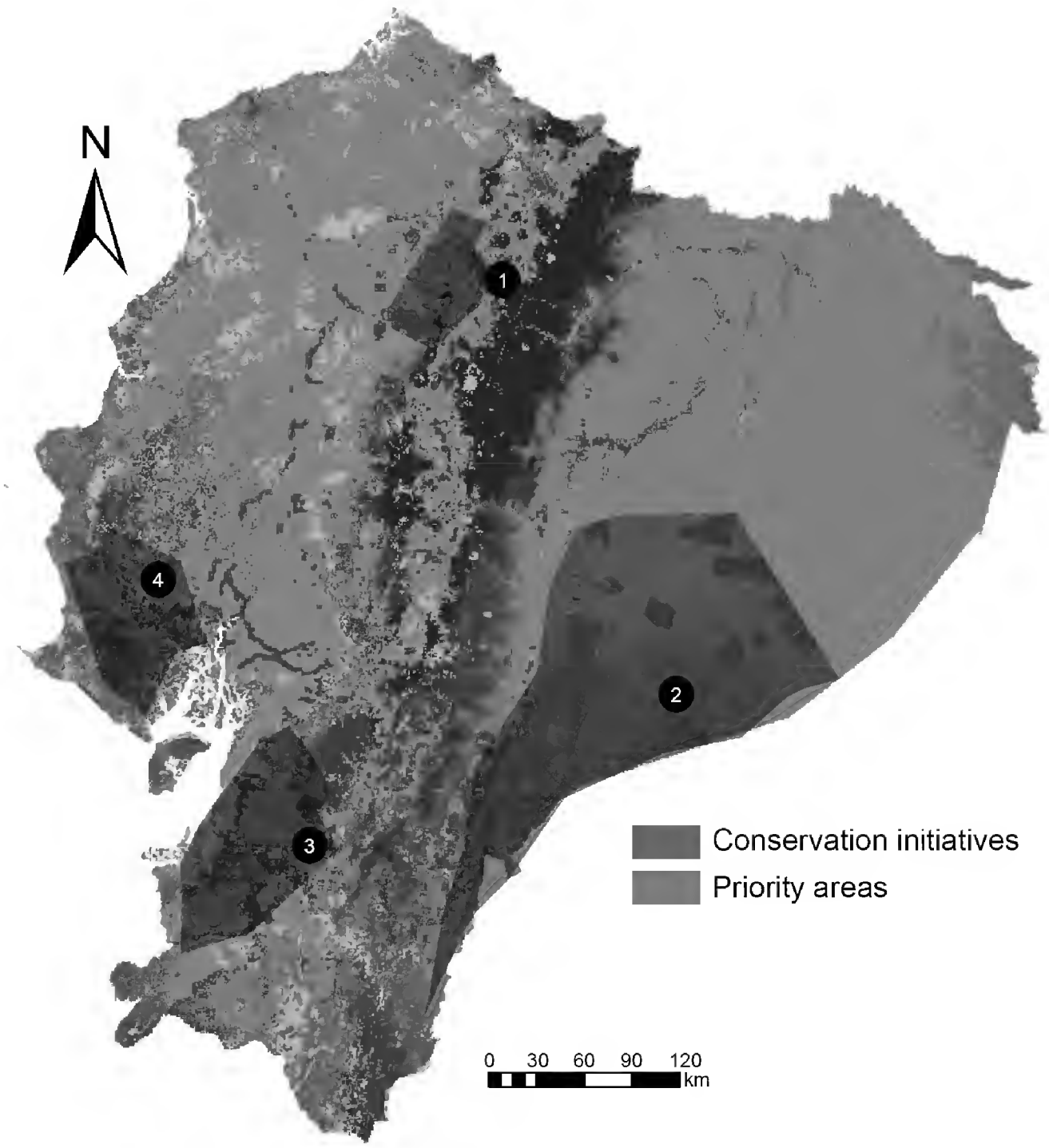

Figure 3. Map of priority areas for the conservation of reptiles in continental Ecuador.

Andean slopes and adjacent lowlands in Azuay and El Oro provinces that include the Molleturo and Mollepungo forests; and (4) the central Pacific coast in Manabí, Santa Elena and Guayas provinces that includes remnant vegetation in the Chongón-Colonche cordillera, as well as SBP areas.

Discussion

With three species per 2,000 km^2, Ecuador is the most reptile-diverse country in the world if country area is accounted for. The highest diversity of reptiles is located in the central and northern Amazon, as well as the Ecuadorian Chocó and adjacent Andean slopes. This pattern of species richness is concordant with other animal and plant taxa, both at local (Lessmann et al. 2014) and continental scales (Bass et al. 2010; Jenkins et al. 2013; Myers et al. 2000), which highlights the biological importance of these areas. Nonetheless, this pattern should not be taken as definitive because a considerable percentage of Ecuador's biodiversity has been discovered in recent years, and not necessarily from the most diverse regions. Nearly 10% of species of reptiles from Ecuador have been described or reported in this century. Of these, nearly 35% were discovered in southern Ecuador, which remains a largely undersampled area that has also been repeatedly identified as an area of conservation priority (this study; Cuesta et al. 2017; Lessmann et al. 2014; Tapia-Armijos et al. 2015).

Unlike other terrestrial vertebrates and plants (González-Palacios et al. 2015; Lessmann et al. 2014; Menéndez-Guerrero and Graham 2013), the conservation status and threats to reptiles from continental Ecuador remain poorly studied. For example, the IUCN Red List of Threatened Species (http://www.iucnredlist.org) lists ~25% of the species of reptiles from continental Ecuador (i.e., excluding the Galápagos islands), of which 17% are Data Deficient. Moreover, recent conservation-planning studies based on a variety of taxa do not include data on reptiles (Lessmann et al. 2016; Lessmann et al. 2014), with only one recent study including 112 species of reptiles for the first time (Cuesta et al. 2017). Here we present the first comprehensive quantitative study of reptile conservation in continental Ecuador including distribution data of nearly 90% of the species of reptiles from continental Ecuador, as well as information on ecosystem protection status and anthropogenic activities that might affect reptile populations negatively.

Table 1. Solutions to identify areas of conservation priority for reptiles from continental Ecuador. Selected solutions are marked with an asterisk.

Solution	Importance	Threat	Opportunity	State protected
A	High	High	yes	yes
B	High	High	no	yes
C*	High	High	yes	no
D*	High	Medium	yes	no
E	High	Medium	no	yes
F	High	Medium	yes	yes
G	Medium	High	yes	yes
H	Medium	High	no	yes
I*	Medium	High	yes	no
J	Medium	Medium	yes	yes
K	Medium	Medium	no	yes
L	Medium	Medium	yes	no

We identified parts of the northwestern slopes of the Andes, central-south Amazonian area, southwestern Andean slopes and adjacent lowlands, and the central Pacific coast as priority areas for the conservation of reptiles in continental Ecuador. These areas partially overlap with some of the Marxan-defined areas reported by Lessman et al. (2014) based on 809 species of amphibians, birds, mammals, and plants; and Cuesta et al. (2017) based on 744 species of amphibians, birds, reptiles (112 species), and plants. Thus, in addition to identifying those areas that are priorities for the conservation of reptiles, our study also supports the conservation of general areas that would benefit a larger number of animals and plants in continental Ecuador. Unfortunately, some of these areas are severely threatened. For example, Tapia-Armijos et al. (2015) reported that ~46% of southern Ecuador's original forests had been converted into pastures and other anthropogenic land cover types by 2008. Similarly, deforestation and extinction in western Ecuador has long been documented (Dodson and Gentry 1991). In conclusion, our study provides further evidence demanding the establishment of protected areas in certain regions of continental Ecuador that remain unprotected and under anthropogenic threat.

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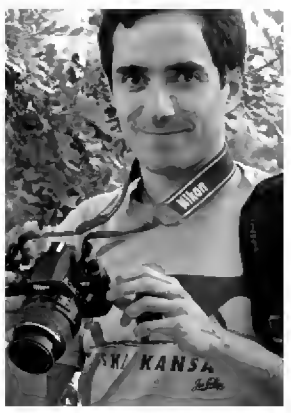

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Appendix 1. Reptile conservation survey: risks, distances, and intensity of threats

1) On a scale from 1 to 10, where 10 is the worst, how bad do you think a primary road is for reptiles?

2) On a scale from 1 to 10, where 10 is the worst, how bad do you think a secondary road is for reptiles?

3) On a scale from 1 to 10, where 10 is the worst, how bad do you think a tertiary road is for reptiles?

4) Imagine that you were to trace a straight line, perpendicular to a road, as far as you think that road has a negative impact on reptiles. How far would you go for a primary road?

0–5 m	10 m	50 m	100 m	500 m	1 km

5) Imagine that you were to trace a straight line, perpendicular to a road, as far as you think that road has a negative impact on reptiles. How far would you go for a secondary road?

0–5 m	10 m	50 m	100 m	500 m	1 km

6) Imagine that you were to trace a straight line, perpendicular to a road, as far as you think that road has a negative impact on reptiles. How far would you go for a tertiary road?

0–5 m	10 m	50 m	100 m	500 m	1 km

7) On a scale from 1 to 10, where 10 is the worst, how bad do you think a mining area is for reptiles?

8) On a scale from 1 to 10, where 10 is the worst, how bad do you think an oil-well area is for reptiles?

9) In your opinion, what is a mine's ratio of negative impact for reptiles?

0–5 m	10 m	50 m	100 m	500 m	1 km

10) In your opinion, what is an oil-well's ratio of negative impact for reptiles?

0–5 m	10 m	50 m	100 m	500 m	1 km

11) On a scale from 1 to 10, where 10 is the worst, how bad do you think livestock husbandry and agriculture is for reptiles?

12) If you were to define a ratio of negative impact for reptiles, where livestock/agriculture facilities represent the center, how far would you go?

0–5 m	10 m	50 m	100 m	500 m	1 km

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Development of in-country live food production for amphibian conservation: The Mountain Chicken Frog (*Leptodactylus fallax*) on Dominica, West Indies

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***Abstract.*—Amphibian populations are in global decline. Conservation breeding programs (CBPs) are a tool used to prevent species extinctions. Ideally, to meet biosecurity, husbandry and other requirements, CBPs should be conducted within the species' geographic range. A particular issue with in-country amphibian CBPs is that of live food supply. In many areas, such as oceanic islands, commonly cultured food species used by zoos throughout the world cannot be used, as escapes are certain to occur and could lead to the introduction of alien, and potentially highly destructive, invasive species. Here, we describe the establishment of live food cultures for the Critically Endangered Mountain Chicken Frog (*Leptodactylus fallax*) at a conservation breeding facility on the Caribbean island of Dominica. Not all invertebrate species were suitable for long-term culture and several species were rejected by captive *L. fallax*, making them unsuitable as food items. Despite the CBP being established within a range state, it was not possible to provide a diet of comparable variety to that of wild *L. fallax*. Our experiences may provide guidance for the establishment of live food culture systems for other conservation breeding programs elsewhere.**

Keywords. Captive breeding, live food culture; invertebrate husbandry, conservation breeding program, Critically Endangered, diet

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Introduction

Amphibian populations are in decline globally, with extinction rates now reaching over 200 times the estimated background rate (Collins 2010; McCallum 2007; Norris 2007). Conservation breeding programs (CBPs) are one of the tools used to mitigate amphibian extinctions (Griffiths and Pavajeau, 2008). In order to be successful, these programs should aim to maintain genetically-representative populations of amphibians in captivity for future conservation translocations (Baker 2007; Browne et al. 2011; Shishova et al. 2011). Establishing amphibian CBPs outside the native range of a species is considered suboptimal due to the risk of transferring novel pathogens to the target species or from the target species into the local environment (Cunningham et al. 2003; Walker et al. 2008; Zippel et al. 2011). Establishing a CBP within the range of the target species reduces this risk, facilitates the provision of natural environmental cycles with relative ease, is often more cost effective and can also instill pride and confidence in the public and other stake holders in the range country (Edmonds et al. 2015; Gagliardo et al. 2008; Tapley et al. 2015a). Amphibian husbandry capacity, however, is often limited in the countries with the most diverse and threatened amphibian faunas (Zippel et al. 2011). For programs in these countries to succeed, it is essential that amphibian husbandry methods, successful or otherwise, are disseminated for the combined benefit of amphibian conservation.

Suboptimal husbandry or nutrition in CBPs can produce maladapted amphibians that are unsuitable for

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release (Antwis and Browne 2009; Mendelson and Altig 2016; Ogilvy et al. 2012). As the nutritional requirements of most amphibians are unknown, suboptimal diets, nutrition, and nutritional disease can be barriers to the implementation of successful amphibian CBPs (Antwis and Browne 2009; Dugas et al. 2013; Gagliardo et al. 2008; King et al. 2010; Ogilvy et al. 2012; Tapley et al. 2015b; Verschooren et al. 2011). Even when the diet is known, it is often not possible to replicate in captivity, as diets for captive amphibians are limited by the commercial availability of food species and the ability to establish breeding colonies of appropriate species, as well as difficulties in providing the prey species themselves with suitable diets. This could have significant repercussions for the success of amphibian CBPs (Tapley et al. 2015a).

The Critically Endangered Mountain Chicken Frog (*Leptodactylus fallax*) is the largest native amphibian species in the Caribbean and one of the world's largest species of frog (Adams et al. 2014; Fa et al. 2010). *Leptodactylus fallax* is endemic to the Caribbean islands of Montserrat and Dominica, although it once occurred on at least five other islands before being lost from those through a combination of habitat loss and degradation, introduced predators, and over-collection for food (Adams et al. 2014; Fa et al. 2010; Malhotra et al. 2007). More recently, the only two extant island populations have been driven towards extinction by the infectious disease, amphibian chytridiomycosis (Hudson et al. 2016a). The population of *L. fallax* on Dominica declined by more than 85% in the 18 months following the first identification of frog mortality due to chytridiomycosis on the island (Hudson et al. 2016a).

In response to these disease-mediated declines on Dominica and Montserrat, a safety net population was established, together with a global partnership, to ensure the survival of *L. fallax* (Hudson et al. 2016b). In 2007, the Zoological Society of London (ZSL), in partnership with the Dominican Forestry, Wildlife and Parks Division, established a captive breeding facility in the botanical gardens of Roseau, the capital of Dominica (Fig. 1A, 1B; Adams et al. 2014; Tapley et al. 2014). A particular issue with regards to the keeping of mountain chickens in captivity is that of food. Mountain chickens have voracious appetites. The commonly cultured food species used by zoos and hobbyists throughout the world could not be used in Dominica as escapees could lead to the introduction of alien (and potentially highly destructive) invasive species onto the island. Therefore, prior to acquiring founding stock of *L. fallax* for the facility, it was imperative to establish live food cultures of sufficient quantity to provide adequate nutrition for the captive animals. Brooks Jr (1982) investigated the diet of *L. fallax* on Dominica and additional prey items were reported by Rosa et al. (2012) for the species on Montserrat. This knowledge was used to inform the species' captive diet.

Herein we describe the methods used to establish sustainable live food cultures for *L. fallax* on Dominica. This may provide guidance for the establishment of subsequent live food culture systems for other range state amphibian conservation breeding.

Methods

Initial considerations

All species selected for culture were harvested from Dominica. Local species were chosen because: 1) accidental release would not lead to introductions of non-native species; 2) acclimatization to local environmental conditions would not be necessary; 3) purchasing and importation costs would be eliminated; 4) availability of stock would not be affected by delayed importation due to tropical storms or other unforeseen circumstances; 5) restocking of depleted cultures would be relatively simple and cost-effective (at the cost of culture adapted species). As well as being local, one of the criteria for choosing a species to trial for live food culture was a perceived ability to rapidly reproduce. Preference was given to those species that had been documented to form part of the wild diet of *L. fallax* (Brooks Jr 1982). In addition to the species initially selected for live food culture, further species were harvested from the wild to include more variation in the captive diet. All substrate was purchased from agricultural suppliers in order to reduce the likelihood of contaminating agents/animals being brought into the facility.

Environmental conditions

The facility in Dominica is open-sided, using a combination of metal wires and mesh netting. This allows the facility to closely match the ambient temperature and humidity of Dominica without the use of climate control methods. The facility itself therefore matches the local temperature range of 20–30 °C throughout the year.

Species used

Since the facility's opening in 2007, live food culture of eight species has been attempted: three species of cricket (*Gryllodes sigillatus*, Fig. 2A; *Gryllus assimilis*, Fig. 2B; *Caribacusta dominica*, Fig. 2C), one cockroach (*Blaberus discoidalis*, Fig. 2D), one beetle (*Zophobas atratus*, Fig. 2E), one slug (*Veronicella sloanii*, Fig. 2F), one snail (*Pleurodonte dentiens*, Fig. 2G), and an assortment of unidentified millipede species (one species represented in Fig. 2H).

Orthoptera

Orthopterans represent a large proportion (44%) of the known diet of *L. fallax* on Dominica (Brooks Jr 1982). Cultures of two cricket species were established at the start of the project: *G. sigillatus* (Fig. 2A), and *C. dominica* (Fig. 2C). A colony of *G. assimilis* (Fig. 2B) was

Fig. 1. (A) The Dominican mountain chicken project captive breeding and research facility, Roseau, Dominica. **(B)** Layout of the conservation breeding facility. *Photo: D. Nicholson.*

formed four years after the facility was set up in order to increase the variety of live food being offered to captive *L. fallax*. The founding population of *C. dominica* was collected from forested areas around the island. *Gryllus assimilis* colonies were established from just two founders that were collected using baited bottle traps. No other individuals of *G. assimilis* have been observed on the island since the original opportunistic encounter. *Gryllus assimilis* and *C. dominica* are native to Dominica and the West Indies (Orthoptera Species File 2016, Weissman et al 2009). *Gryllodes sigillatus* is a southeast Asian native but is now globally distributed (Otte 2006). Individuals used for culture were wild-caught in-country.

Housing: Orthopteran colonies were housed in clear plastic containers measuring 52 × 36 × 38 cm, with an open top covered with fine fly mesh to prevent escape (Fig. 3A). Refugia, including cardboard (hens') egg boxes and cardboard tubes, were provided. Housing containers were cleaned monthly (for *G. sigillatus*) or twice monthly (for *G. assimilus* and *C. dominica*) to remove faecal waste; uneaten food was removed three times per week.

Feeding: Orthopteran colonies were fed fresh food three times per week. A number of different fruits and vegetables were provided, including pumpkin (1 cm cubes), lettuce (diced), cabbage (diced), and carrots (0.5 cm thick discs, halved). Also, a teaspoon each of Seminole Feed® Premium Performance Dog Food (Seminole Feed, Florida, USA) and Pentair® Colour Mix Fish Flake Food (Pentair Aquatic Eco-Systems, North Carolina, USA) were provided to each container three times per week. These were used due to their high protein content (dog food: 26% protein, fish food: 45% protein) and ease of storage.

Breeding: Oviposition sites were created using a 1:1 mix of compacted sand and sphagnum peat moss placed into (10 × 5 × 5 cm) plastic containers (margarine tubs).

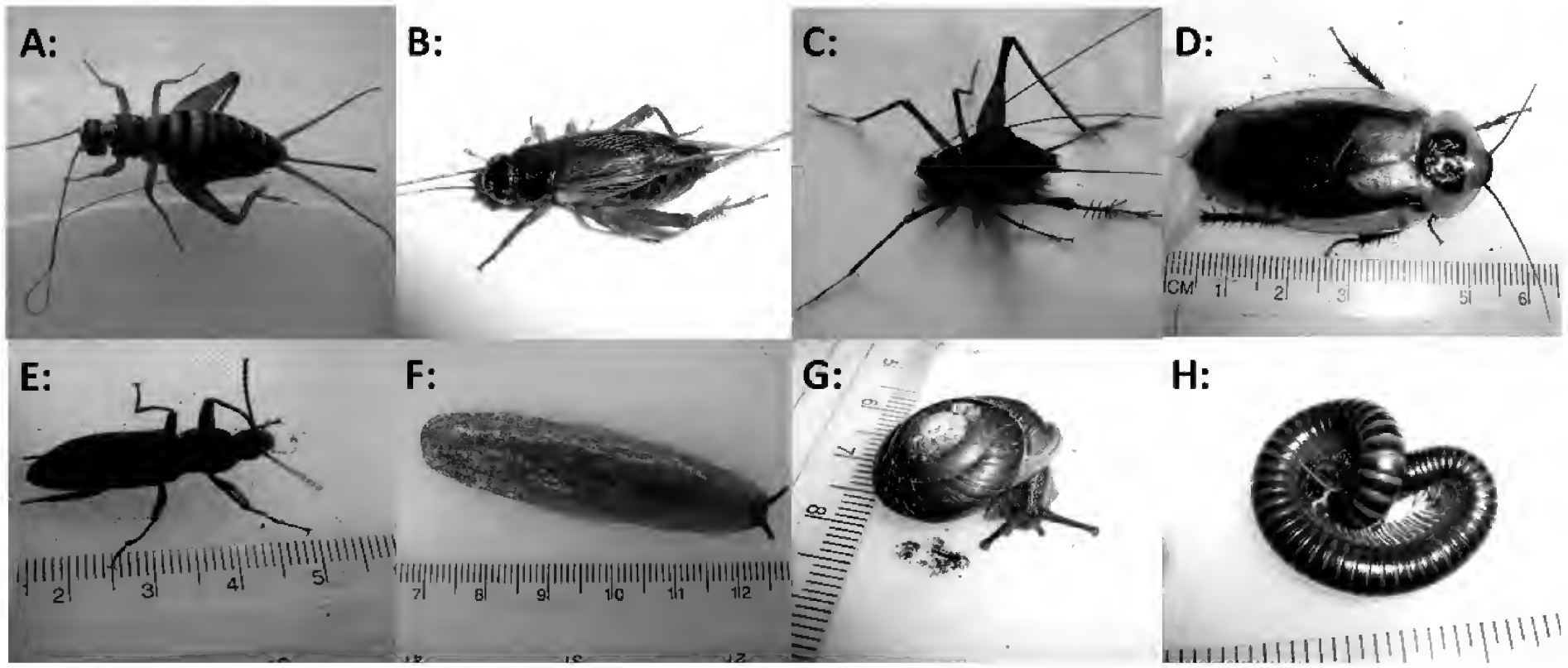

Fig. 2. Cultured species at the CBP in Dominica. **(A)** *Gryllodes sigillatus*. **(B)** *Gryllus assimilis*. **(C)** *Caribacusta dominica*. **(D)** *Blaberus discoidalis*. **(E)** *Zophobas atratus*. **(F)** *Veronicella sloanii*. **(G)** *Pleurodonte dentiens*. **(H)** *Leptogoniulus* sp. *Photos: D. Nicholson.*

These were removed from housing units after two weeks, or sooner if hatchlings were observed (Fig. 3B). After removal, oviposition sites were placed into separate housing units until all 1st instar crickets hatched and exited the nest box. The substrate in the oviposition sites was kept moist at all times.

Rotation: All housing units were arranged and rotated depending on instar. Once the oldest adult crickets had been given sufficient time to lay eggs in the allocated oviposition site and provided with a respite and feeding period, they were fed to the captive *L. fallax* population. The associated oviposition sites were then placed in the first housing unit of the rotation and the remaining crickets at the most advanced stage of development were provided with an oviposition site.

Blattodea

Cockroaches are not known to be a natural prey item for *L. fallax* (Brooks Jr 1982). They were, however, selected for culture due to their durability, high fecundity, large size, suitability to wide scale propagation and because they are readily consumed by captive *L. fallax* in Europe (B. Tapley, pers. obs.). It is not known if *B. discoidalis* (Fig. 2D) is native to Dominica, but it is native to Central America and distributed across the West Indies (Cockroach Species File 2016). The founding stock was collected from a chicken shed on the island.

Housing: Cockroaches were housed in large plastic dustbins (51 × 69 cm) with an open top covered with mesh lining to prevent escape (Fig. 3A). The bins were 1/3 filled with a sphagnum peat moss substrate to facilitate burrowing and cardboard boxes were added as refugia (Fig. 3C). Once per month, the containers were cleaned and the substrate was replaced.

Feeding: Cockroach colonies were fed potatoes (1 cm cubed, approx.), citrus fruits (quartered) and dry dog food (Seminole Feed ® Premium Performance Dog Food) *ad lib*, with fresh food provided three times per week.

Breeding: The substrate used (sphagnum peat moss) provided a sufficient breeding medium.

Coleoptera

Coleoptera comprise 7% of the known diet of wild *L. fallax* (Brooks Jr 1982). Beetles were incorporated into the culture process at the facility after the giant mealworm beetle (*Zophobas atratus*, Fig. 2E) was found to be breeding in the cockroach containers and was noted to be eaten by the captive *L. fallax*. *Zophobas atratus* is native to Central and South America, and it is believed to be naturally occurring in Dominica (Peck 2006). Separate colonies of this beetle were established using the method and housing described above for the cockroaches. Both beetle larvae and adult beetles were offered to *L. fallax*.

Gastropoda

Gastropods make up 18% of the known diet of wild *L. fallax* (Brooks Jr 1982), which have been observed consuming them (D. Nicholson, pers. obs.). Slugs (*V. sloanii*, Fig. 2F) and snails (*P. dentiens*, Fig. 2G) were selected for culture as they are highly abundant and widespread across Dominica, readily observed on nocturnal transects and easy to capture. *Veronicella sloanii* was first discovered on Dominica in 2009 and is believed to have been introduced. *Pleurodonte dentiens* is endemic to Dominica, Martinique, and Guadeloupe (Robinson et al. 2009). Housing: Both gastropod species were housed in clear plastic containers (52 × 36 × 38 cm) with open tops covered with mesh to prevent escape (Fig 3A). All housing

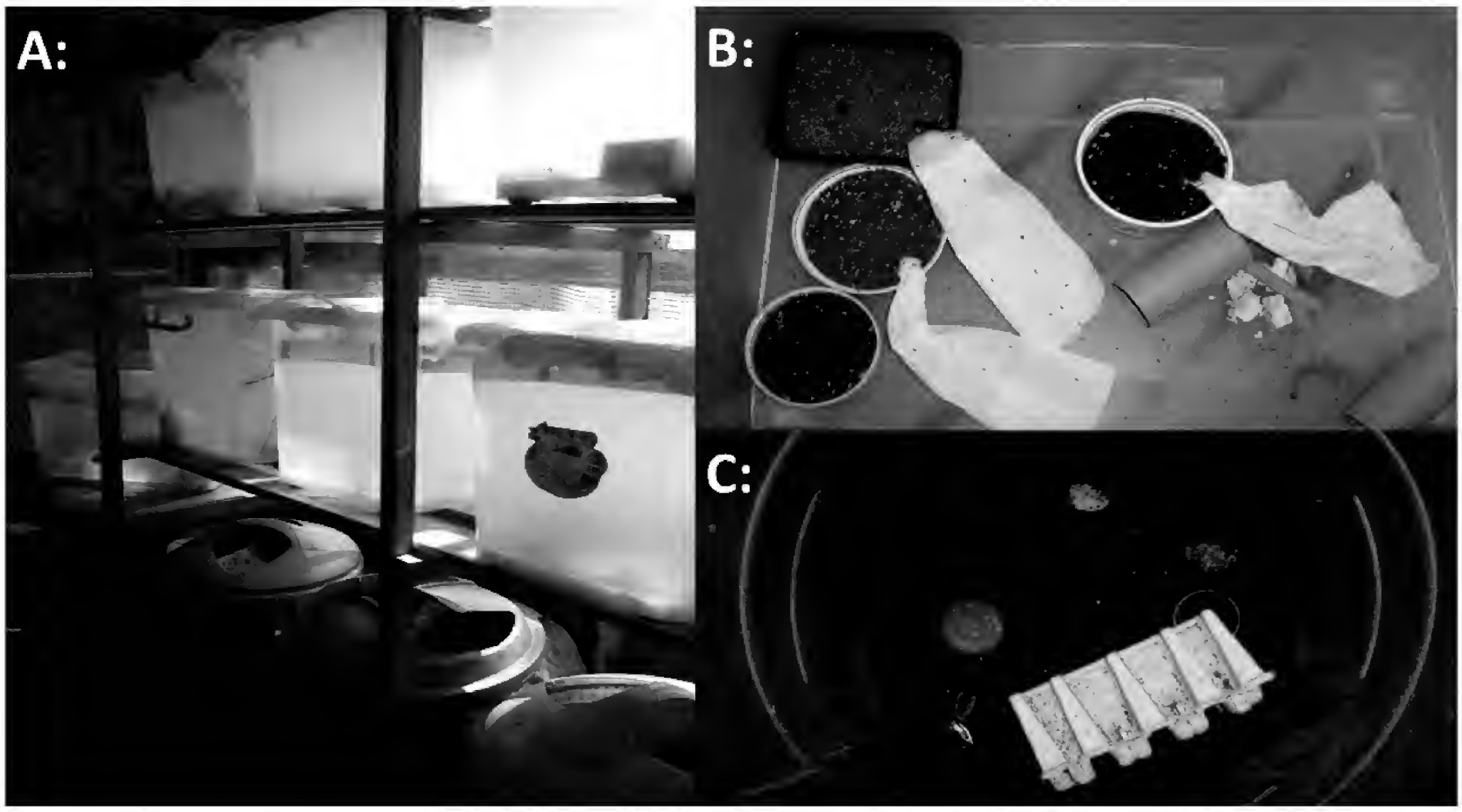

Fig. 3. (A) Two rows of cricket breeding containers and cockroach breeding bins below. **(B)** Inside of a cricket breeding container, including refugia, food items, and several egg laying containers, transplanted into an empty container to allow eggs to hatch. **(C)** Inside view of a cockroach breeding bin, including substrate, refugia, and several food items. *Photos: D. Nicholson.*

units contained refugia such as cardboard egg boxes and sections of tree bark; sphagnum peat moss substrate was also added. Housing containers were cleaned weekly to remove faecal waste and un-eaten food. High humidity was maintained by misting the substrate with water, as required to keep it damp.

Feeding: All gastropod species were fed *ad lib* with the leaves of lettuce, cabbage, and spinach, with fresh food being provided three times per week.

Diplopoda

Millipedes (Fig. 2H) are very common on Dominica and comprise 7% of the known diet of wild *L. fallax* (Brooks Jr 1982). Millipedes were, therefore, chosen for culture at the start of the project but this was soon abandoned as high numbers were readily available in the immediate area of the captive breeding facility. They were, therefore, collected from the wild and presented as a prey source shortly after capture. The different millipede species obtained were not identified to the species level.

Provisioning of *L. fallax*

Up to 11 *L. fallax* were housed in the facility at any one time. The captive *L. fallax* were fed three times per week. Provisioning took place at night as this species is nocturnal (Adams et al. 2014). Night-provisioning increased the likelihood of successful predation and this allowed staff to monitor the behavior, feeding rate, and health of individual frogs. Prey items were placed in a plastic bag and dusted with a multivitamin and mineral supplement high in calcium and containing vitamin D_3 Nutrobal® (Vetark Professional, Winchester, UK) before being released into the frog pens. The amount of prey offered at each feeding event varied depending on the condition of the frogs. Individuals with lower than expected body weight for their size were given more food items to encourage weight gain. Also, before and during the breeding season (February–September, Davis et al. 2000) the number of prey items offered was increased to provide for the additional energy expenditure associated with vocalizing, fighting (males), egg production, and nesting. During this period, 5–6 large prey items (cockroaches) or 10–12 small prey items (crickets) per frog were provisioned. The number of invertebrates offered to the frogs was reduced by 30% during the non-breeding season (October–January).

Preventing metabolic bone disease

Metabolic bone disease (MBD) has been reported in captive *L. fallax* reared on diets supplemented with multivitamin and mineral supplements containing vitamin D_3 and calcium but not provided with ultraviolet B radiation (UV-B) (Tapley et al. 2015b). Animals on the same diet did not develop MBD when provided with UV-B, indicating that the disease was caused by vitamin D_3 deficiency (Tapley et al. 2015b). In most vertebrates, vitamin D_3 is synthesized via exposure to the UV-B present in sunlight. Uptake of ingested vitamin D_3 might not be sufficient in all species for optimal health and this appears to be the case for *L. fallax*. Vitamin D_3 plays a critical role in regulating calcium metabolism, as well as hav-

Table 1. Suitability of invertebrate species captured in the wild on Dominica for live food culture for captive Mountain Chicken Frogs.

Class or Order of live food item	Species of live food item	Sustainable population of food item cultured?	Food item readily consumed by *L. fallax*?
Orthoptera	*Gryllodes sigillatus*	Yes	Yes
Orthoptera	*Gryllus assimilis*	Yes	Yes
Orthoptera	*Caribacusta dominica*	No	Yes
Blattodea	*Blaberus discoidalis*	Yes	Yes
Coleoptera	*Zophobas atratus*	Yes	No
Gastropoda	*Veronicella sloanii*	No	Yes
Gastropoda	*Pleurodonte dentiens*	No	Yes
Diplopoda	*Leptogoniulus* sp.	Yes	No

ing important roles in organ development, muscle contraction, and the functioning of the immune and nervous systems (Wright and Whitaker 2001). To prevent MBD in the captive *L. fallax* all food items were dusted with a multivitamin and mineral supplement which is high in calcium and contains vitamin D_3 (Nutrobal®, Vetark Professional) before being released into *L. fallax* pens. Pens were also supplied with UVB emitting lamps (12% UVB D_3 24 W Basking Lamp, Arcadia).

Results

The ability to develop sustainable invertebrate cultures and the palatability of these as food items for *L. fallax* are summarized for each species in Table 1.

Orthoptera

Gryllodes sigillatus and *G. assimilis* cultures were successful and populations of both species have yielded approximately 50 adults per week to date (over a period of approximately seven and 2 years, respectively). Both species were readily consumed by captive *L. fallax*. However, although readily consumed by *L. fallax*, the live culture of *C. dominica* had a poor outcome. The reproductive output was consistently very low, hatchlings had high mortality rates, and adults had short lifespans. In 2015, five years after its establishment, the population finally collapsed when all surviving adults died without reproducing. The species is very common across Dominica, therefore restarting the culture was not deemed viable due to the ease of collecting animals from the wild and the unsuitability of the species for large scale production.

Blattodea

Live culture of *B. discoidalis* was successful. To date, seven years after its establishment, the facility has maintained a yield of approximately 60 cockroaches per week. This food item was readily consumed by *L. fallax*.

Coleoptera

Giant mealworm beetles were successfully cultured over six years, but consumption rates by *L. fallax* were low. While both life stages of *Z. atratus* were observed to be predated by the captive frogs (D. Nicholson, J. Spencer, pers. obs.), it was noted that adult beetles were promptly regurgitated. Larval forms were almost entirely ignored, apart from a few occasions. The culture of *Z. atratus* was, therefore, discontinued.

Gastropoda

Culture attempts, while successful for both species, yielded low numbers (<10 per week) and were labor intensive: the enclosures required a disproportionate amount of cleaning and maintenance for the yield. Continuous cultures of gastropods were, therefore, stopped after approximately three years. Cultures of both gastropod species are, however, re-established during the breeding season to supplement the diet as they are readily consumed by the captive frogs.

Diplopoda

The harvesting of millipedes was opportunistic, therefore the numbers offered to the frogs as food varied as a result. Despite being consumed by wild *L. fallax* (Brooks Jr 1982), observations of feeding behavior of captive *L. fallax* showed that all millipedes species were regurgitated after ingestion. The use of millipedes as a food item was therefore stopped at the facility. It is possible that the species of millipede provisioned in captivity is different to that observed as a wild food source by Brooks Jr (1982).

Discussion

Provision of an appropriate diet is vitally important for amphibians in CBPs as nutrition influences health, longevity, and reproductive output (Li et al. 2009). The amount of space required for rearing invertebrates for a

relatively small number of frogs was considerable and accounted for 20% of the facility's footprint. When CBPs are conducted in-country, the risk of introduction of alien pest species used as live food is high, especially in island situations. In these cases, a culture of locally-caught species should be developed. A range of such species was trialled in Dominica, of which crickets *G. sigillatus* and *G. assimilis* and the cockroach *B. discoidalis* proved to be most successful. Some other species, such as gastropods, could be cultured successfully, but the labor and other costs of doing so outweighed the ease of harvesting from the wild. Together, the live food culture, augmented by harvesting from the wild, has provided a sustainable supply of food for the maintenance of captive *L. fallax* since their introduction into the facility on Dominica in 2011. Wild harvesting of live food might also provide trace nutrients not obtained from cultured live food, although this was not investigated in our study. The Mountain Chicken Frog CBP on Dominica has had no requirement for the import of food from overseas and no evidence of nutritional disease has been observed, although the frogs have not yet bred in the facility.

The known diet of *L. fallax* in the wild is varied, comprising at least 30 different prey species. In the captive breeding facility on Dominica, however, only five prey species could be regularly provisioned. The depauperate captive diet was primarily due to three reasons: 1) several species were unsuitable for propagation either because of an inability to maintain large enough cultures or because of labor requirements; 2) certain species that could be cultured were not consumed by *L. fallax* in captivity; 3) species not known to be prey items were cultured (including a non-native cricket and cockroach, both of which were already established on Dominica). Even if the known wild diet of *L. fallax* could be matched, the diets used to culture live food are different to those eaten by the invertebrates in the wild. It is unlikely, therefore, that the nutritional content of cultured live food accurately represents that of the same invertebrate species in the wild. It is possible that the cultured diet supplied to the captive frogs is not optimal and therefore a wider range of food species should be harvested from the wild if captive animals are to be maintained and bred on the island in the future. Determining the nutritional content of the wild diet of *L. fallax*, rather than replicating the food items themselves, could inform a viable alternative of manipulating the nutritional content of cultured live food through supplementation or gut loading.

The orthopteran, *C. dominica*, is thought to be one of the key prey items for wild *L. fallax* and is very commonly encountered on Dominica (Brooks Jr 1982); however, we were unable to culture it successfully in large enough numbers to be a useful food item. Possible reasons for the unsuitability of *C. dominica* to the culture process could include inappropriate diet, territoriality, or naturally low reproductive rates. The orthopteran section of the diet therefore relied on two species, *G. assimilis* and *G. sigillatus*, the latter believed to be a non-native species that has become established on Dominica.

A further limitation in our ability to provide a varied diet was the apparent unpalatability of the readily cultured *Z. atratus* and the various unidentified millipede species. These beetles and (certain) millipedes were reported as being key components of the wild diet of *L. fallax* (Brooks Jr 1982), but when offered to captive frogs they were either rejected (millipede sp. and adult *Z. atratus*) or ignored (larval *Z. atratus*). This might be due to the ability of these species to produce defensive chemicals (Gullan and Cranston 2005), which could affect prey preference in captivity in particular because the captive frogs are provided with a readily available food supply. It was not possible to ascertain the identity (even to the level of genus) of the three types of millipede offered as prey items, and only the genus of consumed millipedes was reported by Brooks Jr (1982). Perhaps *L. fallax* is very species-specific regarding millipedes and the wrong prey items were being offered.

The unsuitability of certain invertebrate species as live food items left the facility on Dominica heavily reliant on non-native species which were not listed in the wild diet of *L. fallax* but were easier to culture, notably *G. sigillatus* and *B. discoidalis* (Brooks Jr 1982). *Gryllodes sigillatus* is native to Southwestern Asia but has spread rapidly across the globe and is used in other CBPs where it is non-native (Edmonds et al. 2012). Its arrival date and how well it is established on Dominica is not known. *Blaberus discoidalis* is native to Venezuela, a country which has exported live poultry and other agricultural products to Dominica since establishing a trade relationship in the late 1970s (A. James, pers. comm.; Cockroach Species File 2016). *Blaberus discoidalis* was cultured in the facility after being found in a local chicken coop. As with *G. sigillatus*, the original introduction time frame for *B. discoidalis* is unknown but it is reasonable to suggest the species has been present on Dominica for many years, at least since the trade agreement with Venezuela began.

An accurate replication of the wild diet for animals in CBPs, including those in range states, often is unachievable. For the *L. fallax* CBP, and programs like it, we recommend that the focus should be towards supplying a diversity of locally sourced prey species while, if possible, increasing an understanding of the nutritional make-up of the diet in the wild. It is important to study, wherever feasible, the wild diet of any species maintained as part of a CBP. In this case, comprehensive studies such as Brooks Jr (1982) and additional findings (e.g., Rosa et al. 2012) were important for ascertaining potential prey species for culture. Establishing the wild diet and subjecting this to detailed nutritional analyses should provide the data required to provide an optimal diet in captivity, possibly through manipulating the nutritional content of live food species via supplementation or gut loading.

Conclusion

Sustainable colonies of invertebrates were established using locally caught species on Dominica. These colonies were productive enough to sustain a captive population of *L. fallax*. There was no need to import exotic species to use as live food, but the species most suitable for culture were locally collected, non-native species. The wild diet could not be fully replicated in captivity but frogs did not exhibit any evidence of nutritional disease over the six years of this study.

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